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Determination of the Effects of Material from Alternate Energy Sources on the Upper Respiratory Tract Clearance Mechanism Part I and Part II

Ball State Univ.
Muncie, IN

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DETERMINATION OF THE EFFECTS OF MATERIAL FROM ALTERNATE ENERGY SOURCES ON THE UPPER RESPIRATORY TRACT CLEARANCE MECHANISM

PART I. IN VITRO EXPOSURE TO PARTICULATE POLLUTANTS.

PART II. IN VIVO EXPOSURE TO OZONE.

by

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16. ABSTRACT

Studies were conducted to measure the toxic effects of a variety of substances from the environment on the clearance mechanism of the upper respiratory tract using an in vitro hamster model system.

Studies using hamsters for in vivo exposures to ozone were also conducted to determine the effects of ozone on the cilia beat frequency and cytopathology of the tracheal epithelium.

Organ cultures of hamster tracheal tissue were exposed to graded concentrations of pollutants to determine effects on the respiratory cilia. Paramenters studied were beat frequency, ciliostasis, cytopathology, and ATP concentration.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new manmade environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, nonionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for nonionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This is a two part study conducted to measure the toxic effects of a variety of substances from the environment on the clearance mechanism of the upper respiratory tract using an *in vitro* hamster model system.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

Studies were conducted to measure the toxic effects of a variety of substances from the environment on the clearance mechanism of the upper respiratory tract using an *in vitro* hamster model system.

Studies using hamsters for *in vivo* exposures to ozone were also conducted to determine the effects of ozone on the cilia beat frequency and cytopathology of the tracheal epithelium.

Organ cultures of hamster tracheal tissue were exposed to graded concentrations of pollutants to determine effects on the respiratory cilia. Paramenters studied were beat frequency, ciliostasis, cytopathology, and ATP concentration.

A preliminary survey to determine the "lethal level" and "no-measurable effect level" on ciliary beating for 15 trace metals found in the environment and in the effluents from coal gasification was conducted. The metals toxic at the 100 µg/ml level following 24 and 48 hours of *in vitro* exposure were cadmium, zinc, nickel, manganese, mercury and vanadium. Cobalt was toxic at 10 µg/ml following 48 hours of *in vitro* exposure. Copper was lethal at 10 µg/ml and chromium was lethal at 1 µg/ml after 24 hours of exposure. Extensive and/or complete data for time and dose levels of all the metals was not determined because of the preliminary nature of this survey.

Material from an electrostatis precipitator of a coal fired power plant was not toxic at levels as high as 1075 µg/ml in suspension following 48 hours of exposure. Exposure of rings for 48 hours to a filtrate of 2477 µg/ml also showed no observable toxic effect on the epithelial cells and there was no effect on the beat frequency due to treatment.

In vitro exposures to nickel at 0.63, 6.3 and 63.5 µg/ml for 24, 48, and 72 hours resulted in a dose related pattern of cytopathological changes in the tracheal ring explants. ATP content of nickel treated rings decreased as concentration of nickel increased ($P=0.01$).

Treatment with VO_3 in concentrations of 0.63 µg/ml to 12.5 µg/ml did not produce statistically significant differences in ATP content of rings. However, dose related histological changes resulted from exposure to vanadium in concentrations of 0.63 µg/ml to 12.5 g/ml for 24, 48 and 72 hours. Ciliostasis occurred at levels as low as 0.63 µg/ml and the results were statistically significant from the controls at the $P < 0.005$ confidence level.

In vitro exposure studies using zinc, zinc and ammonium and ammonium ion showed dose response changes in ciliostasis and ATP concentration at levels of 50, 75, 100 µg/ml zinc and 28, 41, 55 µg/ml NH_4. Differences from the controls were statistically significant at $P < 0.05$. A combination of zinc plus ammonium ions was significantly more toxic than either ion alone. All treatment levels of zinc or zinc plus ammonium caused a significant decrease in ATP content as compared to the controls ($P < 0.001$).

Results of *in vivo* exposures to .25 ppm and .5 ppm ozone indicated a dose related reduction in cilia beat frequency that was statistically significant at the $P < 0.01$ level exposed versus control hamsters.

The histological assessment also indicated dose related, adverse, pathological effects in the tracheas of hamsters exposed to ozone.

CONTENTS

Foreword iii
Abstract iv
Figures viii
Tables x
Abbreviations and Symbols. xi
Acknowledgements xiii

PART I - IN VITRO EXPOSURE TO PARTICULATE POLLUTANTS

1. Introduction 1
2. Conclusions 3
3. Recommendations 4
4. Materials and Methods 5
   Animals 5
   Preparation of Tracheal Rings and Organ Culture 5
   In Vitro Exposure 5
   Pollutants. 6
      EP-34 6
      Screening Study. 6
      Nickel 7
      Vanadium 7
      Zinc 7
   Parameters Measured 7
      Determination of Cilia Beating Frequency and % Ciliostasis. 7
      ATP Methodology. 8
      Histological Procedures. 9
      Photomicrography 9
      Statistical Methodology. 9
5. Results and Discussion. 11
   Effects of Exposure to Particulate Pollutants 11
   Effects of Exposure to EP-34. 11
   Screening Study 12
   Nickel 17
      Histological Observation 17
      ATP Measurement. 21
   Vanadium 23
      Histological Observation 23
      Ciliostasis 27
      ATP Measurements 34
   Zinc 35
      Analysis of Ciliostasis Effects. 35
      ATP Analysis 36

PART II. IN VIVO EXPOSURE TO OZONE

1. Introduction 41
2. Conclusions 44
3. Recommendations. 45
4. Materials and Methods. 46
   Animals 46
   In Vivo Exposure. 46
   Preparation of Tracheal Rings 46
   Parameters Measured 48
      Determination of Beat Frequency. 48
      Histological Procedures. 48
      Photomicrography Procedures. 48
      Statistical Methodology. 49
5. Results and Discussion 50
      Beat Frequency 50
      Histological Observations 52

References 58

Bibliography 61

FIGURES

PART I

Number		Page
1	0-hour old untreated ring	17
2	48-hour old untreated ring.	18
3	48-hour old ring treated 24 hours *in vitro* with 0.63 µgNi/ml	19
4	48-hour old ring treated 24 hours *in vitro* with 6.3 µgNi/ml.	20
5	48-hour old ring treated 24 hours *in vitro* 63 µgNi/ml.	21
6	ATP content of rings exposed 24 hours *in vitro* to nickel.	22
7	48-hour old ring treated 24 hours with 1.24 µg V/ml.	24
8	48-hour old ring treated 24 hours with 6.2 µg V/ml	25
9	48-hour old ring treated 24 hours with 24.9 µg V/ml.	26
10	Percent ciliostasis versus time of exposure to four levels of Vanadium 2-month old animals	29
11	Percent ciliostasis versus time of exposure to the level of Vanadium 4-month old animals . .	30
12	Percent ciliostasis versus times of exposure to four levels of Vanadium, 4-month old animals.	31
13	Percent ciliostasis versus log concentration of Vanadium at 24 hours exposure, three studies	32
14	Percent ciliostasis versus log concentration of Vanadium at 48 hours exposure, three studies	33
15	Percent ciliostasis versus time for three concentrations of zinc ion	37

16 Percent ciliostatis versus time for three concentration of zinc plus ammonium ions 38

17 ATP Content of tissues versus zinc and zinc plus ammonium ions concentration 40

PART II

1 *In vivo* exposure facilities 47

2 Ciliary beating frequency versus *in vivo* ozone concentration 51

3 0-hour old untreated ring. 52

4 0-hour ring treated *in vivo* with 0.25 ppm ozone for 3 hours X 3 days 53

5 0-hour ring treated *in vivo* with 0.5 ppm ozone for 3 hours X 1 day 54

6 0-hour ring treated *in vivo* with 0.5 ppm ozone 3 hours X 5 days 55

7 0-hour ring treated *in vivo* with 1.0 ppm ozone, 3 hours X 1 day 56

TABLES

PART I

Number		Page
1	Average beat frequencies of tracheal rings treated with EP-34	11
2	Average beat frequency of tracheal rings treated with the filtrate of EP-34	12
3	Screening Study - Metals which produced no apparent effect.	14
4	Screening Study - Metals which produced toxic effects	15
5	Total ciliostasis of rings treated *in vitro* with Vanadium	28
6	ATP content of rings exposed *in vitro* to ammonium vandate	34
7	Percent ciliostasis versus concentration of zinc ion or zinc plus ammonium ions.	35
8	ATP content of rings treated *in vitro* for 24 hours with zinc and zinc ammonium	39

LIST OF ABBREVIATIONS AND SYMBOLS

PART I

ABBREVIATIONS

ANOVA	--	analysis of variance
bpm	--	beats per minutes
C	--	Celsius
g	--	gram
M	--	molar
mg	--	milligram
ml	--	milliliter
mM	--	millimolar
mm	--	millimeter
N	--	normal
P	--	probability
µg	--	microgram
µm	--	micrometer
x	--	magnification

CHEMICAL SYMBOLS

ATP	--	adenosine triphosphate
$BaCl_2 \cdot 2H_2O$	--	barium chloride
$CdSO_4$	--	cadmium sulfate
CO_2	--	carbon dioxide
$CoCl_2 \cdot 6H_2O$	--	cobalt chloride
Cr^{+3}	--	chromium ion
CrO_3	--	chromium trioxide
CrO_4	--	chromate ion
$CrK(S)_4)_2 \cdot 12H_2O$	--	chromium potassium sulfate
$CuSO4 \cdot 5H_2O$	--	copper sulfate
EDTA	--	ethylenediamine tetracetic acid
EP-34	--	specific samples of particulate matter from coal fired power plant
HCl	--	hydrochloric acid
$HgCl_2$	--	mercury chloride
$MgSO_4$	--	magnesium sulfate
$MnSO_4$	--	manganese sulfate
$NaMoO_4 2H_2O$	--	sodium molybdale
NaOH	--	sodium hydroxide
$NaSO_{+3}$	--	sodium sulfate
NH_4	--	ammonium ion
NH_4VO_3	--	ammonium vandate
Ni	--	nickel
$NiCl_2$	--	nickel chloride
$NiSO_4 \ 6H_2O$	--	nickel sulfate
$Pb(C_2H_3O_2)_2 \ 3H_2O$	--	lead acetate
TRIS	--	trishydroxymethylaminomethane
V	--	vanadium
Zn	--	zinc
$ZnCl_2$	--	zinc chloride

PART II

ppm	--	parts per million
O_3	--	ozone

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Dr. Thad Godish, Dept. of Natural Resources provided the facilities for the exposure and monitoring of the ozone.

The author acknowledges the support and cooperation of Dr. Norman Norton and the Biology Department of Ball State University.

PART I. *IN VITRO* EXPOSURE TO PARTICULATE POLLUTANTS

SECTION I

INTRODUCTION

Airborne particles emmitted into the environment from the utilization of materials from all types of power plants constitute a major source of pollution directly related to the problem of air quality for the human population. A number of new substances from alternate and conventional energy sources have not been toxicologically evaluated for their potential adverse health effects in humans. Some of these substances, including the ones in this study, are materials derived from shale oil, coal gasification and liquefaction, and particulate effluents from both mobile and stationary sources.

Once these substances are inhaled and deposited within the lung, they may exert a toxic effect on the host via one or more of these mechanisms: (1) the substance may be intrinsically toxic due to its inherent chemical or physical properties, (2) the substance may interfere with one or more of the clearance mechanisms in the respiratory tract, and (3) the substance may act as a carrier of an absorbed toxic substance.

Depending on their site of deposition, these substances come into intimate contact with very specialized host cells, i.e., cilia and alveolar macrophages, whose function is to rid or clear the body of inhaled substances. If these defense systems of the host are reduced in activity then an accumulation of both viable and nonviable substances would occur, which in turn might jeopardize the health of the host.

The overall purpose of this project was to screen a variety of substances from the environment for their toxic effect on the clearance mechanism of the upper respiratory tract using an *in vitro* hamster model system. In testing for cytotoxicity, an *in vitro* organ culture system was used employing hamster tracheal rings to determine specific effects on normal ciliary activity and ciliated epithelium. This isolated pseudostratified ciliated epithelium provided a controlled means for assessing some direct effects of the pollutants.

Samples of pollutants tested were supplied by the contractor or the EPA Project Officer. Materials tested and studies completed in this project include the following: a screening study using 15 trace elements from the environment, cytohistological and ATP effects of nickel, a beat frequency study of a sample from an electrostatis precipitator and two in depth studies of the toxicity of vanadium, and zinc/ammonium sulfate ion.

Dose response experiments were designed using various concentrations and lengths of exposures. The various parameters assayed in the hamster tracheal model included: histopathological alterations, ciliary beat frequency, ciliostasis, and ATP determinations.

SECTION 2

CONCLUSIONS

The purpose of this project was to evaluate the effects of various materials on the ciliary function of the upper respiratory tract. Experiments completed in this project demonstrate that alterations in ciliary function seem to be indicative of an impairment of the normal clearance mechanism. The results from the data showed statistically significant differences between control and exposed tracheal tissue in an *in vitro* organ culture model.

Data from the screening study of 15 trace metal pollutants indicated toxicity to tracheal ciliary beating activity for mercury, zinc, cadmium, vanadium, nickel, cobalt, copper and chromium. Molydenum, manganese, barium, lead, chromium (in the +3 valence state), sulfite, and magnesium were not toxic after 48 hours of *in vitro* exposure to concentrations of at least 100 µg/ml.

Histologically, there was evidence of damage to the ciliated epithelium of the tracheal rings following exposure to graded concentrations of nickel and vanadium. Denudation, tufting of cilia, sloughing, blebbing, vascularization of cytoplasm, notched nuclei, eosinophilic inclusions and flattened cells were observed in a dose related pattern in tracheal rings exposed to either pollutant.

Treatment with both nickel and zinc sulfate or zinc ammonium sulfate resulted in statistically significant ($P < 0.05$) dose related decreases in the ATP content of tracheal ring explants. *In vitro* exposure to vanadium did not produce correlating changes in the ATP content of rings.

In terms of tracheal clearance, the significant differences in % ciliostasis with both zinc and vanadium indicate impairment of normal ciliary activity. If the effectiveness of the mucociliary activity depends on normal functioning of the ciliated epithelium of the upper respiratory tract, it follows from the data presented that inhaled environmental pollutants can inhibit the normal mucociliary function needed to clear possible harmful and infectious agents from the respiratory tract.

SECTION 3

RECOMMENDATIONS

The *in vitro* hamster tracheal ring model used in this study is excellent for preliminary screening of environmental chemicals. This *in vitro* system is a convenient and inexpensive means of examining the effect of acute and short term exposure to potentially toxic substances. Such substances, if present in sufficient quantities to allow for testing, should be examined by preliminary screening in such an *in vitro* system. Previous studies (1,2) which compare this *in vitro* method with *in vivo* exposures support the use of the *in vitro* model for testing the effects of pollutants on the living animal.

In addition, the short term, acute exposures of the materials evaluated in this study should be extended to include multiple exposures and recovery projects. Pollutants should be evaluated in combination with a stress factor such as exercise. Further detailed studies should include *in vivo* exposures for validation of *in vitro* results.

Our study of ATP concentration was an attempt to elucidate the site of pollutant effect on ciliary activity. Further studies might examine oxidative and enzymatic responses to pollutants.

SECTION 4

MATERIALS AND METHODS

ANIMALS

Male and female Syrian Golden hamsters (outbred Lak: LVG (SYR) Charles River Lakeview, Wilmington, MA) weighing 90 to 160 g were used in these studies. Hamsters of this weight are considered young adults and are two months old or older. Animals of one sex were housed in groups of three per cage, and provided food and water *ad libitum*.

PREPARATION OF TRACHEAL RINGS AND ORGAN CULTURE

The method used for maintaining the tracheal rings as bio-indicators of metal toxicity has been previously described by the author (1,2). Trachea were excised aseptically, washed in Eagle's basal medium (Gibco, Grand Island, NY), and cut into rings approximately 1 mm thick. Twelve tracheal rings were usually obtained from each animal. The rings were placed in individual 50 x 9 mm wettable plastic petri dishes with 2.5 ml of Eagle's basal medium with Earle's Salts. The medium was supplemented with 10% fetal calf serum, 1% penicillin (10,000 units per ml) - streptomycin (10,000 mg per ml), 0.5% l-Glutamine (200 mM) and 1% mycostatin (10,000 units per ml). The pH of the medium was adjusted to 7.4 with 1 N NaOH. The rings were incubated for 24 hours at 37° C, 5% CO_2 and 90% relative humidity to allow for adjustments to the *in vitro* culture environment.

IN VITRO EXPOSURES

The selected pollutants were dissolved in culture medium at the desired maximum concentration, then serially diluted with control medium to produce the graded concentrations used for *in vitro* exposures. The solutions were stirred for two hours using a magnetic stirrer, and then filtered through a 0.2 mm millipore filter. The exact concentrations of the metal solutions were determined using a Perkin Elmer Model 306 atomic absorption spectrophotometer. The pH of all media was maintained at 7.4 $\pm$ 0.1 by the addition of 1N NaOH or 1 N HCl as required (1,2).

Following the initial 24-hour incubation in maintenance medium, the organ culture medium was replaced with 2 ml of fresh medium containing the pollutant. Rings were exposed to graded concentration of the pollutant for various time periods during incubation at 37°C, 5% CO_2, and 90% relative humidity. Rings were examined at 24 hour intervals.

POLLUTANTS

EP-34

Tracheal rings were exposed in in vitro to graded dose levels of a suspension and a filtrate of EP-34. EP-34 was obtained from the Environmental Protection Agency. It is characterized as a 3.3 µm or smaller sized fraction of an electrostatic precipitator ash from a coal burning power plant. During incubation in the EP-34 suspension, the organ culture dishes were placed on a rocker platform. Concentrations of the EP-34 suspension ranged between 1.1 and 1075 µg/ml. EP-34 filtrate concentrations of 1239 and 2477 µg/ml were used. Tracheal ring ciliary beating frequency was measured to determine the toxicity of both the EP-34 suspension and the filtrate.

Screening Study

Preliminary studies were conducted to find dose level responses of a number of trace chemicals that might possibly be found in the effluents of alternate energy sources. From a list of potential pollutants from coal gasification, fifteen compounds were chosen that were soluble in the culture medium used. The individual chemicals were tested in duplicate at three or more concentrations in the culture medium. The purpose was to find a "lethal level" and a "no measurable effect level" on ciliary beating frequency. The fifteen compounds tested were: vanadium (NH_4VO_3), molybdenum ($NaMoO_4.2H_2O$), barium ($BaCl_2,2H_2O$), lead ($Pb(C_2H_3O_2)_2$), mercury ($HgCl_2$), chromium as CrO_4^{-2} (CrO_3^{-}), chromium as Cr^{+3} ($CrK(S)4)2.12H_2O$), zinc ($ZnCl_2$), nickel ($NiSO_4.6H_2O$), copper ($CuSO_4.5H_2O$), cobalt ($CoC_2.6H_2O$), manganese ($MnSO_4$), sulfur as SO_2($NaSO_3$), magnesium ($MgCl^2$), and cadmium ($CdSO_4$).

After examining the results of this screening study, three of the metals were selected for in-depth evaluation. These three metals were nickel, vanadium and zinc.

Nickel

Tracheal rings were exposed *in vitro* to various concentrations of nickel chloride ($NiCl_2$) dissolved in sterile Eagle's basal medium. Concentrations of nickel ranged between 0.63 and 63 µg/ml and the effects of these concentrations on appearance of tracheal ring tissue and ATP content were examined.

Vanadium

Tracheal rings were exposed *in vitro* to various concentrations of ammonium vanadate (NH_4VO_3 Fisher) dissolved in sterile Eagle's basal medium. Concentration of vanadium ranged between 0.62 and 24.9 µg/ml. The effects of this pollutant were measured on tracheal ring ciliary beating, ATP content, and histopathology.

Zinc

Tracheal rings were exposed *in vitro* to various concentration of zinc sulfate, zinc ammonium sulfate, and ammonium sulfate. Zinc sulfate (Mallinkrodt AR), zinc ammonium sulfate (either stochiometric amounts of the separate salts from Mallinkrodt or the double salt prepared by Pfaltz & Dauer), and ammonium sulfate (Mallinkrodt AR) were weighed and dissolved in sterile Eagle's basal medium at concentration of 300 µg/ml zinc (Zn) ion and/or 165 µg/ml ammonium (NH_4) ion. When necessary, pH was adjusted with one normal sodium hydroxide. The media were then diluted 1:3, 1:4, or 1:6 to obtain the desired concentration of 100, 75, or 50 µg Zn/ml and/or 55, 41 or 28 µg NH_4/ml. The effects of zinc and ammonium ions, separate and combined, on ciliary beating and ATP content were examined.

PARAMETERS MEASURED

Determination of Cilia Beating Frequency and % Ciliostasis

Following the initial 24-hour incubation that allowed for adjustment to the culture environment, all tracheal rings were examined for normal ciliary movement and/or evidence of cytopathology with an inverted microscope at a magnification of 250X or 640X. If beat frequency effects were being studied, the base-line frequency for each ring was determined at this time by the use of an electronic stroboscope (General Radio Strobotac Type 1531-AB). A Nicholson Precision Instruments air curtain was used to maintain the temperature at 37°C while determing the cilia beating frequency. Beats per minute were counted in each quadrant of the ring, and their average was recorded as the initial frequency in beat per minute (1).

To determine % ciliostasis each ring was visually divided into theoretical quadrants. Each quadrant was then assigned a numerical value to indicate degree of ciliostasis: 0.0 for total ciliostasis, 0.5 indicating that some but not all cilia in the quadrant were beating, and 1.0 for all cilia beating.

The values for all quadrants were combined to give a sum value for that ring. This value was then divided by 4, the total beating value possible for a ring, to obtain the maximum beating value for that ring. This beating value, expressed as a percentage, was then subtracted from 100 percent beating to give the percent ciliostasis for the total ring.

ATP Methodology

ATP measurements of the rings were determined using a Turner fluorometer with the UV source blocked off and a Bausch and Lomb VOM-5 strip chart recorder. One milliliter of firefly extract reconstituted with distilled water and diluted 1:2 with ATP diluent was placed in a cuvette in the fluorometer. The recorder was started and the standard or sample was injected as the tracing crossed a line on the chart paper. After a 15 second delay, the response was recorded for 1 minute then later integrated with a planimeter. Standard curves were prepared for each day's analysis using triplicate samples of 0.05 µg, 0.10 µg, and 0.15 µg ATP. The data for the standards were fitted to a straight line using a least squares procedure. The standard line was then used to calculate the concentration of ATP in the samples. ATP concentration was expressed as µg ATP per mg fresh (wet) tissue weight.

The disodium salt of adenosine triphosphate (ATP) isolated from equine muscle (99.5% pure) was obtained from Sigma Chemical Co., as was the lyophilized extract of firefly tails. TRIS (trishydroxymethylaminomethane) was obtained from Brae Laboratories. Disodium EDTA (ethylenediamine tetraacetic acid) and magnesium sulfate were from Mallindrodt, AR grade.

ATP standards were prepared by making a 1000 µg/ml solution in a modification of the diluent recommended by Dupont (4), containing 0.01 M TRIS, pH 7.7, 2.46 g $MgSO_4$, and 0.372 g $EDTA\text{-}Na_2$. Aliquots of this standard were stored frozen until used. At the time of each analysis, an aliquot of the ATP standard was thawed and diluted 1:100 with the ATP diluent. This solution was used as a reference standard for the determinations. Tracheal rings were removed from the medium, gently drained on Kimwipes, and weighed to the

nearest 0.01 mg. Each ring was placed in a tube containing 1.00 ml TRIS buffer (0.01 M, pH 7.7) in a boiling water bath. ATP was extracted for 10-15 minutes, the tubes containing the rings were then immediately placed on ice until analysis. Reproductability studies in this laboratory showed that ATP was stable for at least 30 minutes in the water bath, and at least 4 hours on ice.

Histological Procedures

Trachael rings were processed for light microscopy in Bouin's solution at 0 hour or after in vitro culturing periods of 24, 48 or 72 hours in maintenance or treated media. The rings were dehydrated in three changes of 50% ethanol followed by three changes of dioxane. The rings were infiltrated in three changes of Paraplast Plus in vacuo at 60° C, the final change always lasting overnight. After embedding in fresh Paraplast Plus, tissues were sectioned at 4 µm and stained with Harris' hematoxylin and eosin.

Photomicrography Procedures

Photographs of the control and treated sections were taken using an American Optical Microstar Series 10 microscope with an AO Expostar shutter control. All photographs were taken at a magnification of 1000X (oil).

Both black and white prints, color prints, and transparencies were taken using the following films: Polaroid black and white (Polaroid Type 665P/N), 35-mm color (Kodak Photomicrography Film #2483, and Ektachrome Tungsten 50).

Statistical Methodology

The data were analysed by the analysis of variance (ANOVA) program, BMDP2V, from the Health Science Computing Facility at the University of California at Los Angeles. Analyses was made for possible effects due to the different treatment materials, sex of the animal, treatment level, and their possible interactions. Where no effect was seen (between the various series of treatments, and between the sexes) the data were pooled and the ANOVA rerun. Where significant effects were seen, Scheffe's test (5,6) was used to find the significant differences. For the tests between materials at a given level a hybrid error term (7) was used. As a conservative estimate for the degrees of freedom, the lower of the two degrees of freedom of the two error terms in the

hybrid was used. Two or three rings from each hamster were treated at each concentration to reduce the variability from animal to animal, and each ring thus served as its own pretreatment control in the beat frequency and % ciliostasis studies.

Data were available from the beat frequency counting procedure that allowed for a semiquantification of the percent ciliostasis (cessation of beating), each ring had been arbitrarily sampled at four different locations each time it was counted. These locations were approximately 90° apart, giving the ring a division into quadrants. All quadrants for a given concentration were summed to give a total sample. If a quadrant had all cilia beating its value was given as 1, if total ciliostas had occurred in the quadrant a value of zero was assigned, and if some cilia was beating but some not a value of 0.5 was assigned. A Chi-square analysis, was performed to compare the effects of the separate ions and in the case of zinc and ammonium, the combination at a given concentration level, and Cochran's Q-test (6) was used to compare the response between dose levels and for the response over time for a given ion or combination. The McNemar test was used for pairwise comparison between levels within a treatment and time, or time periods within a treatment level. It is equivalent to Cochran's Q-test for two contrasts. (7,8)

SECTION 5

RESULTS AND DISCUSSION

EFFECTS OF EXPOSURE TO PARTICULATE POLLUTANTS

In vitro studies were conducted to examine the effects of various particulate pollutants on hamster tracheal rings. Rings were exposed to graded concentrations of EP-34, nickel, vanadium, and zinc in addition to 15 compounds used in a screening project. Parameters measured were beat frequency and/or ciliostasis, ATP content and histopathology.

EFFECTS OF EXPOSURE TO EP-34

There were no observable toxic effects either to the epithelial cells or the beat frequency after 48 hours of exposure. The beat frequencies at each concentration of suspended EP-34 are reported in Table 1. As can be seen, the measured values did not vary at any one time period by more than 30 beats per minute, which is less than the size of several of the standard deviations.

TABLE 1 AVERAGE BEAT FREQUENCY OF TRACHEAL RINGS TREATED WITH EP-34

(Average of 4 rings ± the standard deviation)

Dose Level µg/ml	Before Treatment	24 Hours After Treatment	48 Hours After Treatment
Control	1186 ± 32	1209 ± 19	1175 ± 17
1.1	1200 + 35	1207 ± 35	1187 ± 16
10.8	1183 ± 10	1199 ± 28	1198 ± 15
108	1204 ± 12	1225 ± 7	1204 ± 18
1075	1200 ± 16	1208 ± 31	1178 ± 11

The results of treatment with the filtrate of EP-34 suspended in medium are reported in Table 2. In this table it is apparent that there was no alteration in the beat frequency, therefore, no statistical analysis was performed.

TABLE 2 AVERAGE BEAT FREQUENCY OF TRACHEAL RINGS TREATED WITH THE FILTRATE OF EP-34

(Average of 4 rings ± the standard deviation)

Treatment Level µg/ml	Before Treatment	24 Hours After Treatment	48 Hours After Treatment
Control	1214 ± 23	1168 ± 7	1087 ± 23
1239	1206 ± 4	1170 ± 18	1085 ± 7
2477	1206 ± 12	1189 ± 34	1084 ± 34

SCREENING STUDY

The objective for this screening study was to conduct a preliminary evaluation of some particulates occurring in the effluent from coal gasification in order to determine their effect on the ciliated epithelium of the hamster's trachea. This was not intended to be a detailed study, but a simple and fast procedure for selecting samples for in depth study. The parameters selected to indicate possible effects of these metals were ciliary beat frequency and necrosis of the tissue.

Initially, dose levels of 1, 10, and 100 ppm for all the metals were selected to establish a no effect low dose. Higher dose levels were either randomly selected (subjectively) or based on toxicity levels reported in the literature. At the highest levels, cessation of ciliary beating and total necrosis of the tissue was evident for a number of the pollutants tested.

Initially eight tracheal rings from one hamster were used for each metal tested. The metals were tested in duplicate at three concentrations (1, 10, and 100 µg/ml) and compared to two control rings. The rings were incubated in the media containing the pollutent and in the maintenance medium (controls) for 24 hours. The only parameters measured were beat frequency and condition of rings (normal or necrotic). If no effect was apparent at even the highest concentration, the rings were exposed for a second 24-hour period and beat frequency of the rings exposed to the highest concentration of the metal was again measured.

The results of all concentrations tested for all the metals are given in Tables 3 and 4.

The metals that apparently were not toxic in 48 hours at dose levels of 100 µg/ml were molydenum, barium, lead, chromium (in the +3 valence state), sulfur, magnesium and manganese. Results are given in Table 3. In addition, or instead of the three dose levels of 1, 10, and 100 µg/ml, some of these metals were tested at higher concentrations.

When lead produced no toxic effects (no change in beat frequency) at 100 µg/ml level, three higher doses were tested: 126, 251, and 502 µg Pd/ml. Even this higher concentration produced no effects in 96 hours when compared to the controls. This lack of lead toxicity may be due to precipitation of the lead by phosphate in the medium. The medium turned cloudy when lead acetate was added.

Chromium (+3), sulfite, magnesium and manganese were also tested at concentrations greater than 100 µg/ml. However, no toxic effects were evident unless very high dose levels and/or long exposure periods were used.

Results of those metals producing effects in 48 hours at concentrations of 200 µg/ml or less are presented in Table 4. Vanadium, cadmium, nickel, cobalt, copper, chromium (as chromate) all produced decreased ciliary beating frequency as compared to controls and/or necrosis of tissue at dose levels of 100 µg/ml or less in 48 hours. Vanadium and chromium produced decreased beating frequency and/or necrosis in 48 hours at dose level of less than 2 µg/ml. Copper and cobalt produced decreased ciliary beating frequency or necrosis after 48 hours of 10 µg/ml. Mercury caused necrosis of tissue after 48 hours exposure to 133 µg/ml and zinc after 48 hours of exposure to 201 g/ml.

Based on these preliminary results, experiments were conducted using vanadium, zinc, and nickel. Parameters studied for vanadium included pathology and ciliostasis. Parameters studied for zinc included ciliostasis and ATP determinations. Based on the result of a previous study (2), pathology and ATP determinations were the only parameters studied for nickel.

TABLE 3. SCREENING STUDY - METALS WHICH PRODUCE NO APPARENT EFFECT AT DOSE LEVELS OF 100 µg/ml. CILIA BEATING FREQUENCY (MEAN AND STANDARD DEVIATION) OF TRACHEAL RINGS TREATED IN VITRO WITH VARIOUS METALS.

DOSE µg/ml	LENGTH OF EXPOSURE (HOURS) 24	48	72	96
	MOLYBDENUM $NaMoO_4.H_2O$			
Control	907± 60.1(2)*			
1	875± 7.1(2)			
10	915± 21.2(2)			
100	1032± 46.0(2)	918(1)		
	BARIUM $BaCl_2.H_2O$			
Control	1015± 84.9(2)			
1	904± 15.6(2)			
10	994± 69.3(2)			
100	972± 40.3(2)	878(1)		
	LEAD $Pb(C_2H_3O_2)_2.3H_2O$			
Control	972± 50.1(4)	924± 44.8(2)	1078±236.9(2)	928±102.5(2)
1	972± 3.5(2)	1038± 53.0(2)	835(1)	1040(1)
10	1032± 10.6(2)	965± 84.9(2)	910±141.4(2)	920± 49.5(2)
100	1018± 60.1(2)	852±109.6(2)	860(1)	
126	930± 28.3(2)			
251	1004± 12.7(2)			
502	938± 53.0(2)	905(1)		
	CHROMIUM (Cr^{+3}) $CrK(S)_4)2.12H_2O$			
Control	982±103.9(2)	894± 65.1		
125	964± 90.5(2)	901± 19.1(2)		
250	982± 75.7(2)	902± 10.6(2)		
500	972± 12.0(2)	899± 1.4(2)		
	SULFUR (SO_2) $NaSO_3$			
Control	987± 66.8(16)	981±109.5(16)	1001±164.0(16)	1012±125.2(8)
60	1010± 88.0(9)	1056± 64.9(9)	1118±118.2(9)	1035±122.3(9)
75	966± 53.1(6)	898±126.7(6)	854±114.3(6)	
150	954± 88.2(6)	895±188.1(6)	866± 79.7(6)	
300	1003±111.8(6)	822± 98.0(6)	825± 75.1(6)	
600	1017± 72.2(11)	1059± 71.3(9) 2 of 11 nectrotic	8 of 9 necrotic	
	MAGNESIUM $MgCl_2$			
Control	1110±106.1(2)	997± 81.3(2)	980± 84.9(2)	930± 56.6(2)
50	1097± 31.8(2)	1051± 54.4(2)	982± 10.6(2)	945± 49.5(2)
500	1120± 42.4(2)	1000± 56.6(2)	1000± 35.4(2)	870± 49.5(2)
5000	1082± 31.8(2)	2 nectotic		
	MANGANESE $MnSO_4$			
Control	1077± 64.1(4)	934± 47.8(4)	882±110(4)	889± 50.3(4)
6	1152± 24.7(2)	947±130.8(2)	922± 3.5(2)	940± 42.4(2)
60	1105±113.1(2)	887± 3.5(2)	890± 63.6(2)	802± 88.4(2)
600	1080± 56.6(2)	962± 24.7(2)	2 necrotic	

*Numbers in parentheses indicate number of rings

TABLE 4 - SCREENING STUDY - METALS WHICH PRODUCED EFFECTS AT DOSE LEVELS OF 200 µg/ml.
CILIA BEATING FREQUENCY (MEAN AND STANDARD DEVIATION) OF TRACHEAL RINGS TREATED IN VITRO WITH VARIOUS METALS

DOSE	LENGTH OF EXPOSURE (HOURS)			
µg/ml	24	48	72	96
		VANADIUM NH_4VO_3		
Control	969± 63.7(41)*	937± 61.1(38)	886±181.0(25)	885± 34.6(3)
0.62	998± 60.1(20)	904±105.6 (11 of 38 necr)**	889± 70.3 (1 of 16 necr)	792± 69.1(4)
1.24	993± 51.0(19)	857± 83.5 (2 of 18 necr)	895± 71.1 (2 of 14 necr)	764±114.5(3)
2.49	966± 50.6(12)	896± 50.7 (5 of 12 necr)	(3 of 4 necr)	
3.1-3.37	940± 30.6(13)	788±132.3 (5 of 12 necr)	(2 of 3 necr)	
6.2-6.74	949± 79.9(12)	(10 of 13 necr)	(2 necr)	
17.4-13.48	960± 50.2(13)	(8 of 10 necr)	(2 necr)	
24.9-26.96	941± 69.3(13)	(9 of 13 necr)	(2 necr)	
		CADMIUM $CdSO_4$		
Control	1194± 41.0(2)	1090± 84.9(2)	862± 17.7(2)	1005± 28.3(2)
1	1095± 35.4(2)	835±247.5(2)	895(1)	1055(1)
10	1040± 7.1(2)	970±117.4(2)	795±205.1(2)	(2 necr)
100	1045± 77.8(2)	(2 necr)		
		NICKEL $NiSO_4 \cdot 6H_2O$		
Control	1132± 67.2(2)	867± 46.0(2)	880± 21.2(2)	952± 60.1(2)
1	1030± 99.0(2)	822±109.6(2)	790± 49.5(2)	896± 49.5(2)
10	1072± 74.2(2)	860± 77.8(2)	896± 58.7(2)	
100	943±144.3(2)	(2 necr)		
		COBALT $CoCl_2 \cdot 6H_2O$		
Control	1035± 63.6(2)	937± 17.7(2)	950± 56.6(2)	882± 81.3(2)
1	1166± 58.7(2)	877± 31.8(2)	878± 40.3(2)	877± 29.6(2)
10	1085± 91.9(2)	860± 99.0(2)	848± 26.2(2)	(2 necr)
100	1142± 24.7(2)	(2 necr)		

* Numbers in parentheses indicate number of rings.

** necr - necrotic

Table 4 (Cont.)

DOSE	LENGTH OF EXPOSURE (HOURS)			
µg/ml	24	48	72	96
		COPPER $CuSO_4 \cdot 5H_2O$		
Control	1142± 38.9(2)	877± 46.0(2)	990± 21.2(2)	912± 74.2(2)
1	1107± 53.0(2)	945±113.1(2)	890± 77.8(2)	971± 22.6(2)
10	1165± 28.3(2)	(2 necr)		
100	1150± 14.1(2)	(2 necr)		
		CHROMIUM ($CrO_4^=$) CrO_3		
Control	1092± 46.0(2)	917± 74.2(2)	810± 70.7(2)	972± 46.0(2)
	1072± 76.4(2)	(2 necr)		
11.2	1121±122.3(2)	(2 necr)		
112	1065± 99.0(2)	(2 necr)		
		MERCURY $HgCl_2$		
Control	970± 49.5(2)	1037± 17.7(2)	945± 91.9(2)	917± 46.0(2)
1.3	900± 21.2(2)	955± 56.6(2)	941±210.0(2)	Contaminated 860
13.3	970± 77.8(2)	955± 63.6(2)	967±166.1(2)	852± 24.7(2)
133	1025± 56.6(2)	(2 necr)		
		ZINC $ZnCl_2$		
Control	1027± 46.0(2)	985± 49.5(2)	865±106.1(2)	895± 70.7(2)
2.0	1025± 28.3(2)	937± 38.9(2)	867± 46.0(2)	875(1)
20.1	1015± 42.4(2)	855± 35.4(2)	865± 77.8(2)	754± 29.7(2)
201	1022± 3.5(2)	(2 necr)		

NICKEL

Histological Observations

Control Rings - Changes Due to In Vitro Tissue Culturing

The histological appearance of the ciliated epithelium of a freshly isolated untreated ring shown in Figure 7 is that of a typical pseudostratified, columnar, ciliated epithelium. The cilia appear even in length, straight, and thick with no gaps. The oval shaped nuclei of the columnar cells are approximately centered in the cell vertically, and the nuclei of the basal cells are close to the basement membrane. Cytoplasm is uniform in appearance.

Figure 1. 0-hour old untreated ring, hematoxylin and eosin, 1000X. Note the even, thick cilia, the pseudostratified arrangement of columnar and basal cells.

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After 48 hours in maintenance medium, Figure 2, the height of the epithelian layer is reduced so that the cells appear cuboidal rather than columnar. The cilia are still even in length, straight and thick. Nuclear size does not change, but the shape changes slightly from an oval to an almost circular shape (9 x 5 µm to 7 x 6 µm). During this time, the relative position of the nucleus moves higher in the cell, and the nucleus fills a greater proportion of the cell.

Control rings at 48 hours in our system showed some changes such as sloughing of cells. We interpret these changes as due to reduced nutrition of the basement membrane. In the intact animal blood vessels located beneath the basement membrane provide nutrient diffusion across a 3-5 µm thickness to the epithelial cells, and especially to the basal cells. In a closed culture system, only the ciliated cells are in direct contact with the medium and it seems likely that the basal cells would receive limited nutrients and oxygen through diffusion across the 20 µm thick cell layer. Thus some cells are sloughed during the culture period.

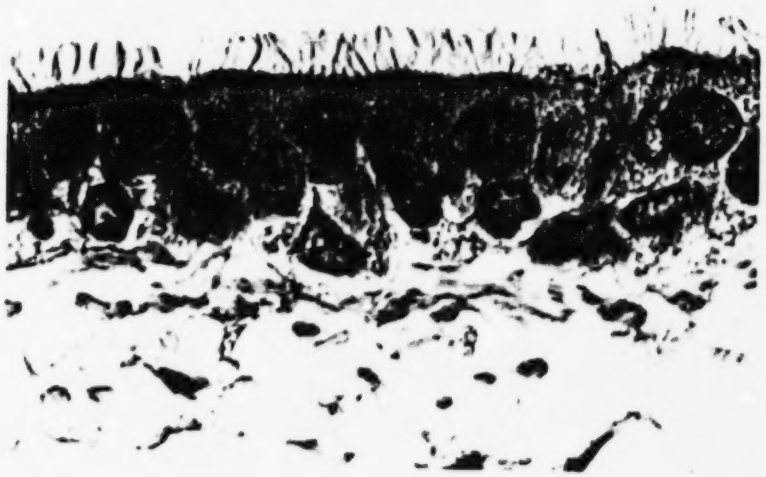

Figure 2. 48-hour old untreated ring, hematoxylin and eosin, 1000X. Note reduced cell height when compared to 0-hour control. Cilia even, straight, and thick. This is the control for comparing to a 48-hour old ring, treated 24 hours in graded doses of nickel.

Changes Due to Treatment with 0.63 μgNi/ml --

Changes occurring after 24 hours of *in vitro* exposure to 0.63 μgNi/ml are shown in Figure 3. Cilia are less regularly arranged. Some cells show cloudy swelling.

Figure 3. 48-hour old ring treated 24 hours *in vitro* with 0.63 μgNi/ml, hematoxylin and eosin, 1000X. Cilia are less evenly arranged and not as dense as in control rings. Note also areas of cloudy swelling and cytoplasmic basophilic inclusions.

Changes Due to Treatment with 6.3 μgNi/ml --

Pseudostratified arrangement of cells is less orderly than in the 0.63 μgNi/ml treated rings. Cilia also losing their orderly arrangement. Some areas show loss of cilia (Figure 4).

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Figure 4. 48-hour old rings treated 24 hours in vitro with 6.3 µgNi/ml, hematoxylin and eosin, 1000X. Cilia and cellular arrangement showing less order than in 0.63 µgNi/ml treated rings.

Changes Due to Treatment with 63 µgNi/ml --

Epithelial layer showing extensive disruption due to treatment with 63 µgNi/ml (Figure 5). Some areas have total loss of cilia. Pyknotic nuclei present, cloudy swelling and vesicle present in cytoplasm. Much blebbing and sloughing of cells.

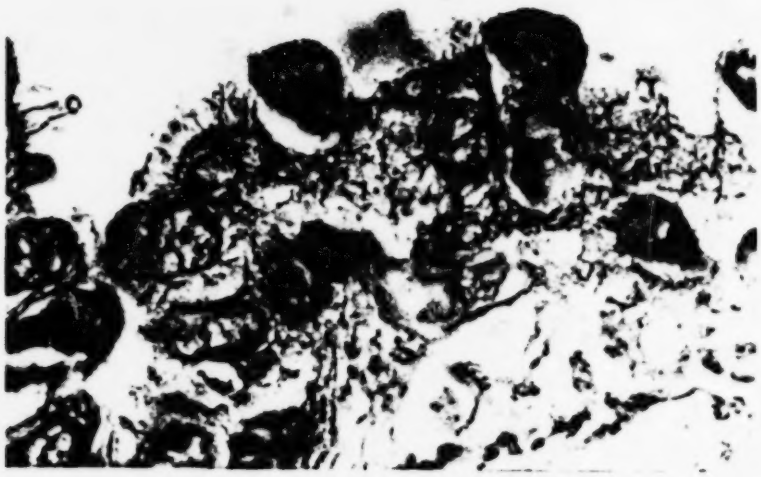

Figure 5. 48-hour old ring treated 24 hours *in vitro* with 63 µgNi/ml, hemotoxylin and eosin, 1000X. Epithelial layer totally disrupted. Many pyknotic nuclei, cytoplasm vesicular, much loss of cilia.

ATP Measurements

The results of *in vitro* exposure to 10, 20, and 40 µgNi/ml are presented in Figure 6. ATP content decreased as concentration of Ni increased. Analysis of variance indicates a highly significant decrease ($P=0.01$) due to treatment. There was no significant difference due to sex of animals or interaction of sex and treatment.

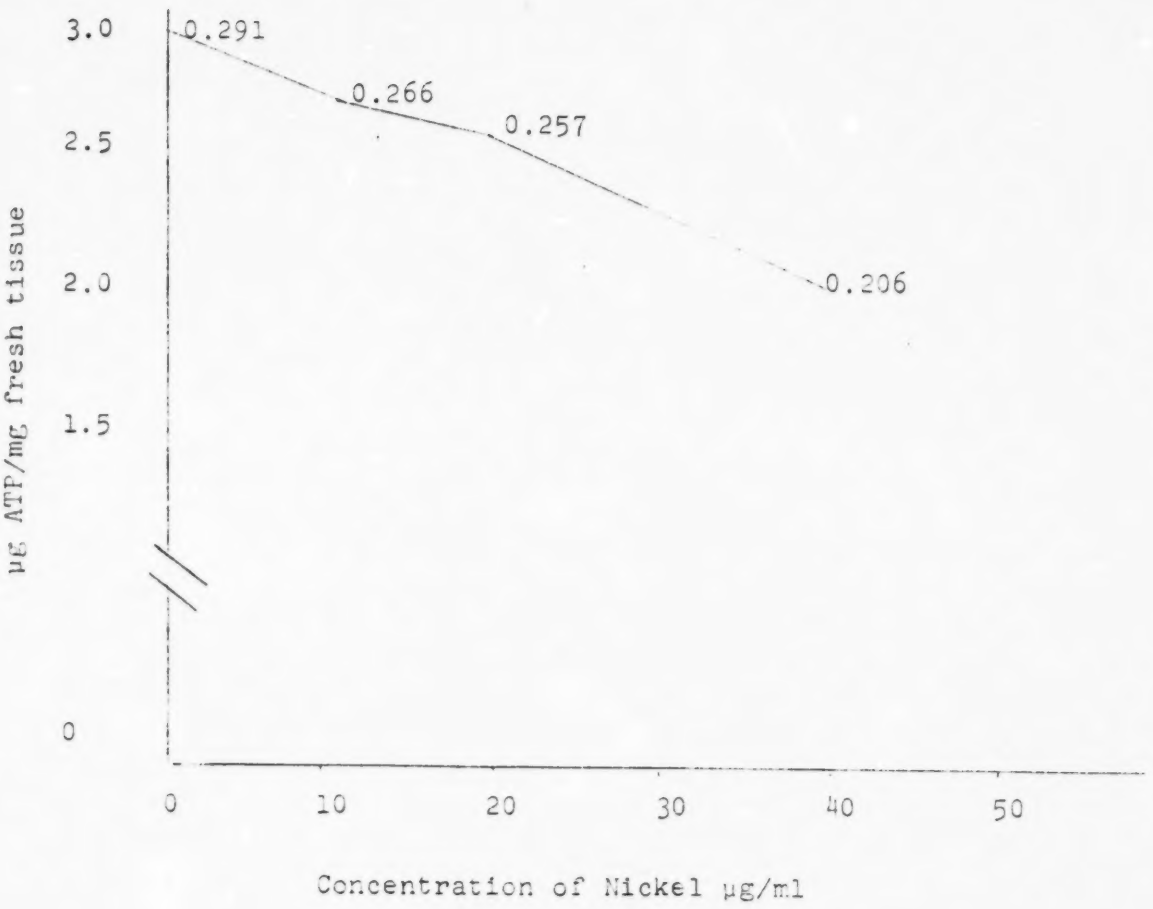

Figure 6. ATP content of rings exposed 24 hours in vitro to nickel.

VANADIUM

Histological Observations

Control Rings - Changes Due to In Vitro Tissue Culturing --

Description of changes in control rings occurring in the in vitro culturing are given previously in the Nickel study. See Figure 1 and 2.

Changes Due to Treatment with 0.62 µg/ml Vanadium --

Treatment with 0.62 µg/ml vanadium as NH_4VO_3 causes a range of changes in the cells at the end of 24 hours of treatment.

The epithelium is flattened compared to controls with nuclei near the cell surface. Blebbing and sloughing occur with denudation and tufting. A few vesicles form especially in the cytoplasm of basal cells. The nuclei are more irregular in shape and show condensation of the chromatin. The edges of some of the nuclei are notched and grooved as if the nuclear membrane has started to fold inward. The chromatin condenses into granules around the edge of the membrane and then fragment. At this concentration of vanadium no changes occur that could be correlated with length of treatment.

Changes Due to Treatment with 1.24 µg/ml Vanadium --

After rings were treated with 1.24 µg/ml for 24 hours (Figure 7), the tissue layer appears disorganized, with cells and nuclei no longer in any arrangement. Cytoplasm appears disrupted with the formation of vesicles.

Nuclear shape is irregular with notches and grooves more pronounced than at .62 µg/ml. Nuclei are nearer the cell surface and the chromatin has condensed into a few hyperchromic granules in many cells. Cilia are tufted and many cells are denuded. These effects are more prominent at 48 and especially at 72 hours. Cells are swollen after 72 hours with blebbing, and there is evidence of cell sloughing. The tissue layer is flattened. Eosinophilic inclusions are present at 72 hours.

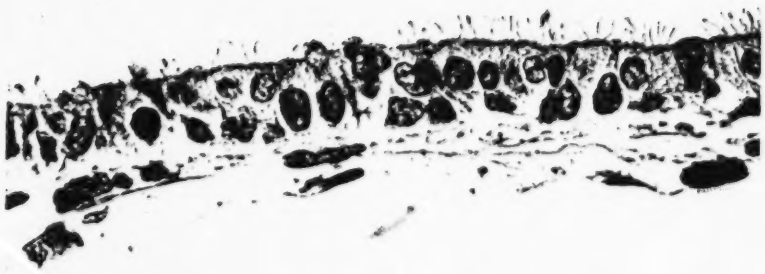

Figure 7. 48-hour old ring treated for 24 hours with 1.24 µgV/ml, hematoxylin and eosin, 1000X. Note the loss of pseudostratification, the irregular shaped nuclei, the clumping and unevenness (tufting) of cilia. Some denudation and blebbing and sloughing are occurring. Cytoplasmic vesicles are present (hydropic degeneration).

Changes Due to Treatment with 3.1 µg/ml and Higher Concentration of Vanadium --

After 24 and 48 hours of treatment with 3.1 µg/ml, the effects seen with 1.24 µg/ml treatment are more pronounced. Blebbing is present and all cells appear swollen. The pseudostratification is gone. Vesicles are present in the cytoplasm and their number increases with time. Eosinophilic inclusions are present at 24 hours. Nuclei are

irregular in size and the chromatin is either condensed or fragmented into large irregular granules in many of the cells. Nuclei are present at the surfaces of cells. Notching is prominent in nuclei at 24 hours and more pronounced at 48 hours. Cilia show tufting and cells are denuded. Following 48 hours of treatment at this level, the rings are considered dead with total ciliostasis, blebbing, denudation, and sloughing of cells.

Figure 8 shows severely damaged tissue after treatment of 6.2 µg/ml for 24 hours. The difference in effects between 6.2 and 12.4 µg/ml treatment levels is one of degree of damage. The cells are swollen, blebbed, and sloughed. Large vesicles are prominent. The tissue varies from columnar to squamous around the tracheal ring. Eosinophilic inclusions were present. Nuclei are irregular in shape and are either pyknotic or karyorrhectic. Notching and grooving of the nuclei are evident. Nuclear position in the cell is highly variable. Cilia are tufted and cell denudation is extensive. Similar results are seen after exposure to 12.4 µg/ml for 24 hours.

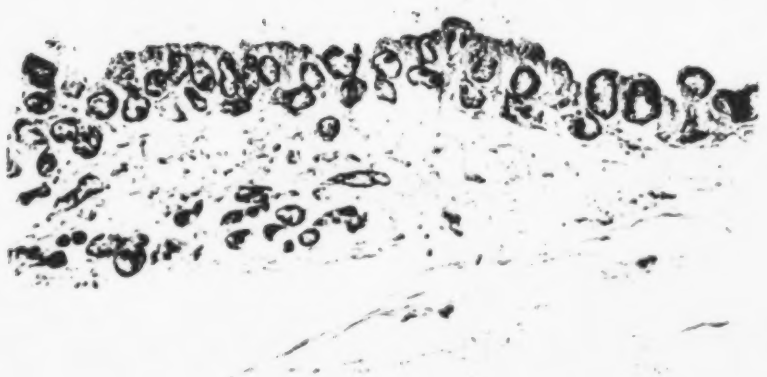

Figure 8 48-hour old ring treated for 24 hours with 6.2 µg/ml, hematoxylin and eosin, 1000X. Note disorganization of tissues, denudation and swelling. Considerable blebbing and sloughing, nuclei are irregularly shaped with chromatin clumping. What cilia are present are totally disordered. Vesicles are present in many cells.

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Treatment with 24.9 µg/ml (Figure 9) causes a complete destruction of the cilial layer within 24 hours. Most cells are swollen, sloughed, and contain vesicles. Nuclei are pyknotic or karyorrhectic. Cells contain eosinophilic inclusions. Cilial organization is totally lacking.

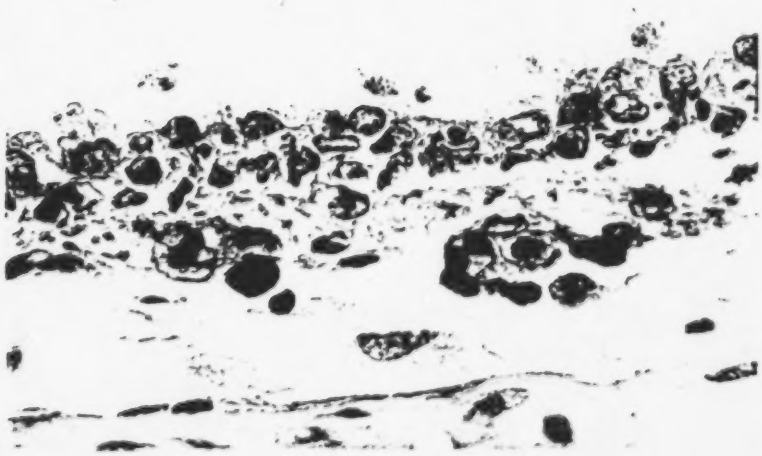

Figure 9.. 48-hour old ring treated 24 hours with 24.9 µg V/ml, hematoxylin and eosin, 1000X. Note total destruction of epithelium with vesicles, pyknosis, karyorrhexis, cloudy swelling, blebbing, sloughing, and fragmentation of cells and nuclei.

Ciliostasis

Because of the reduction in cilia beating amplitude in rings treated with vanadium, it was difficult to accurately measure beat frequency with the stroboscope. The ciliostatic effects of *in vitro* exposure to vanadium was studied on three different age groups: 2-, 4-, and 7-month old hamsters. The extent of ciliostasis follows a dose and time related pattern for all three age groups of animals. The cumulative percent of ciliostasis for each of the three groups is reported in Table 5 and graphed in Figures 10-12. From Table 5, it appears that after 24 hours of treatment at doses above 3.37 µg vanadium/ml there is 100% ciliostasis for all rings from all three age groups. At 3.37 µg/ml and below, the percent of ciliostasis increases with the increasing dose levels. For a given dose level the percent ciliostasis increases with time. For all but the two lower dose levels (0.62-0.67 and 1.24-1.35 µg V/ml) ciliostasis is nearly 100% by 48 hours. This pattern of ciliostasis holds true for all studies despite variability differences in animal age, animal batch and differences in sensitivity to vanadium.

In the ciliostasis determinations, a difference does appear in comparing the 4-month old animals to the 2- and 7-month old animals. The 4-month old animals (Figure 11) are more sensitive to vanadium. The differences in sensitivity are more apparent in Figures 13 and 14 where the percent ciliostasis is plotted against the log of vanadium concentrate. A Chi-square analysis shows that at 2.7 µg/ml the 7-month old animals respond most like the 4-month old group. However, for all other concentrations and times tested, the 7-month old and 2-month old groups are similar. In all cases, the 4-month old animals came from a separate order. Therefore, there is the possibility of variability due to age and/or batch of animals.

TABLE 5. TOTAL CILIOSTASIS OF RINGS DUE TO VANADIUM TREATMENT

Concentration Vanadium (µg/ml)	% total ciliostasis cumulative at 24, 48, & 72 hours after treatment		
2-month old animals (4)*			
	24 hrs.	48 hrs.	72 hrs.
24.9	100%	100%	100%
12.4	100%	100%	100%
6.2	100%	100%	100%
3.1	61%	97%	100%
2.5	62%	97%	100%
1.24	24%	42%	N.D.[a]
0.62	12%	18%	N.D.
Control	0%	0%	N.D.
4-month old animals (4)			
2.50	92%	100%	100%
1.25	75%	100%	100%
0.63	50%	72%	87%
Control	0%	0%	3%
7-month old animals (8)			
26.96	100%	100%	100%
13.48	100%	100%	100%
6.74	100%	100%	100%
3.37	100%	100%	100%
2.70	88%	100%	100%
1.35	44%	63%	80%
0.67	14%	31%	41%
Control	0%	1%	1%
Overall (all ages - 16)			
3.5	100%	100%	100%
3.1-3.4	82%	99%	100%
2.5-2.7	83%	99%	100%
1.24-1.35	46%	67%	87%
0.62-0.67	23%	38%	56%
Control	0%	0%	1%

(*number of animals given in parentheses)

[a] N.D. - Not Determined

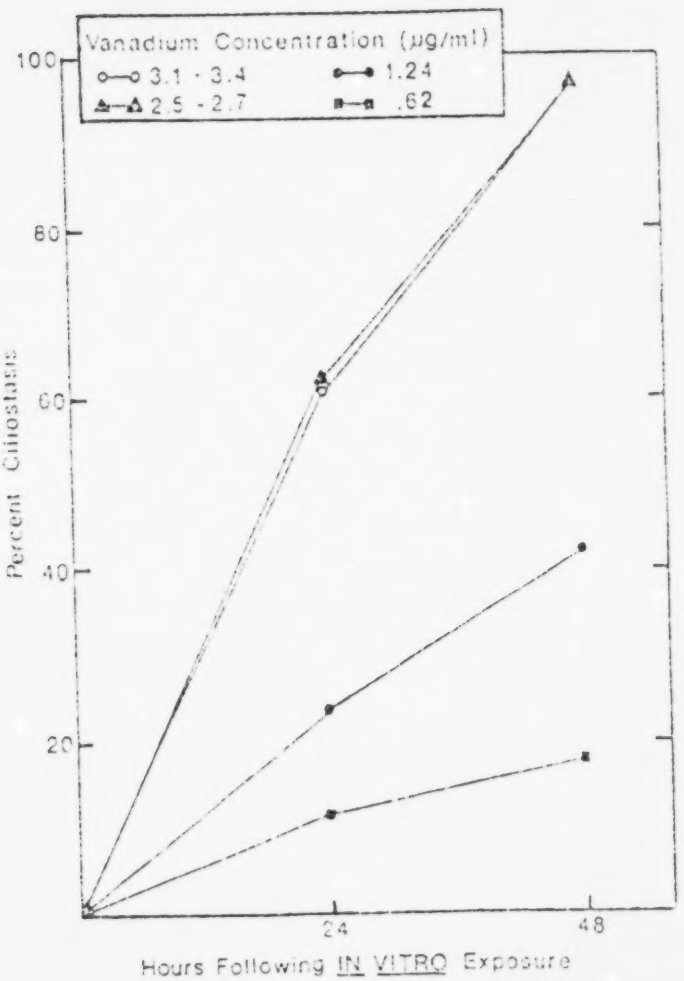

Figure 10. Percent Ciliostasis versus time of exposure to four levels of Vanadium, 2-month old animals.

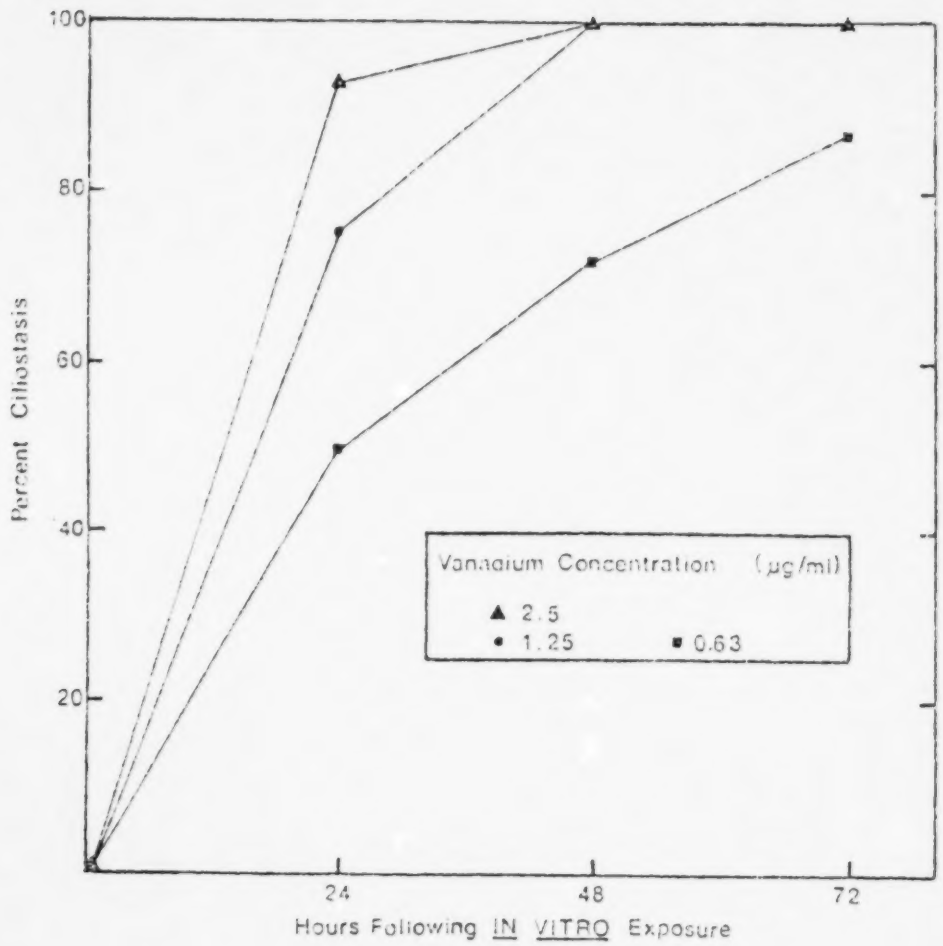

Figure 11. Percent Ciliostasis versus time of exposure to three levels of Vanadium, 4-month old animals.

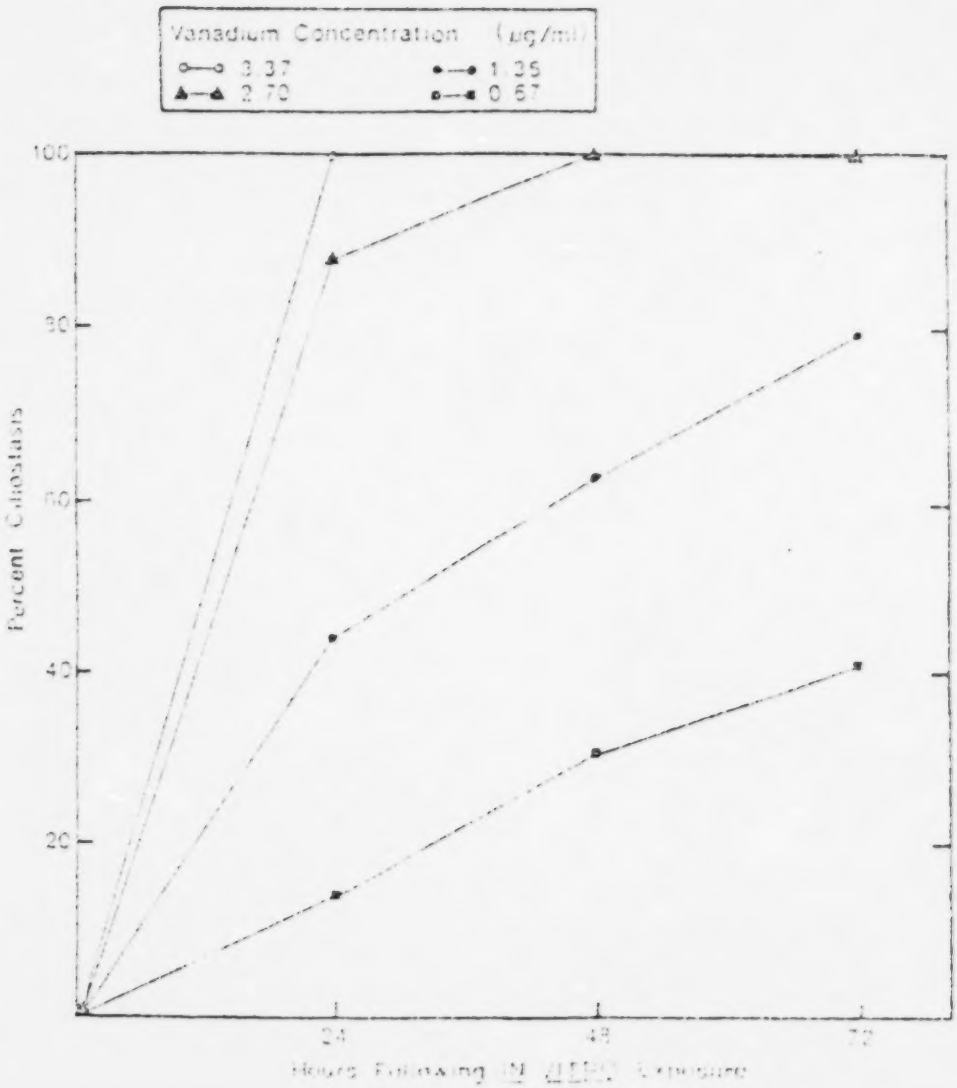

Figure 12. Percent Ciliostasis versus time of exposure to four levels of Vanadium, 7-month old animals.

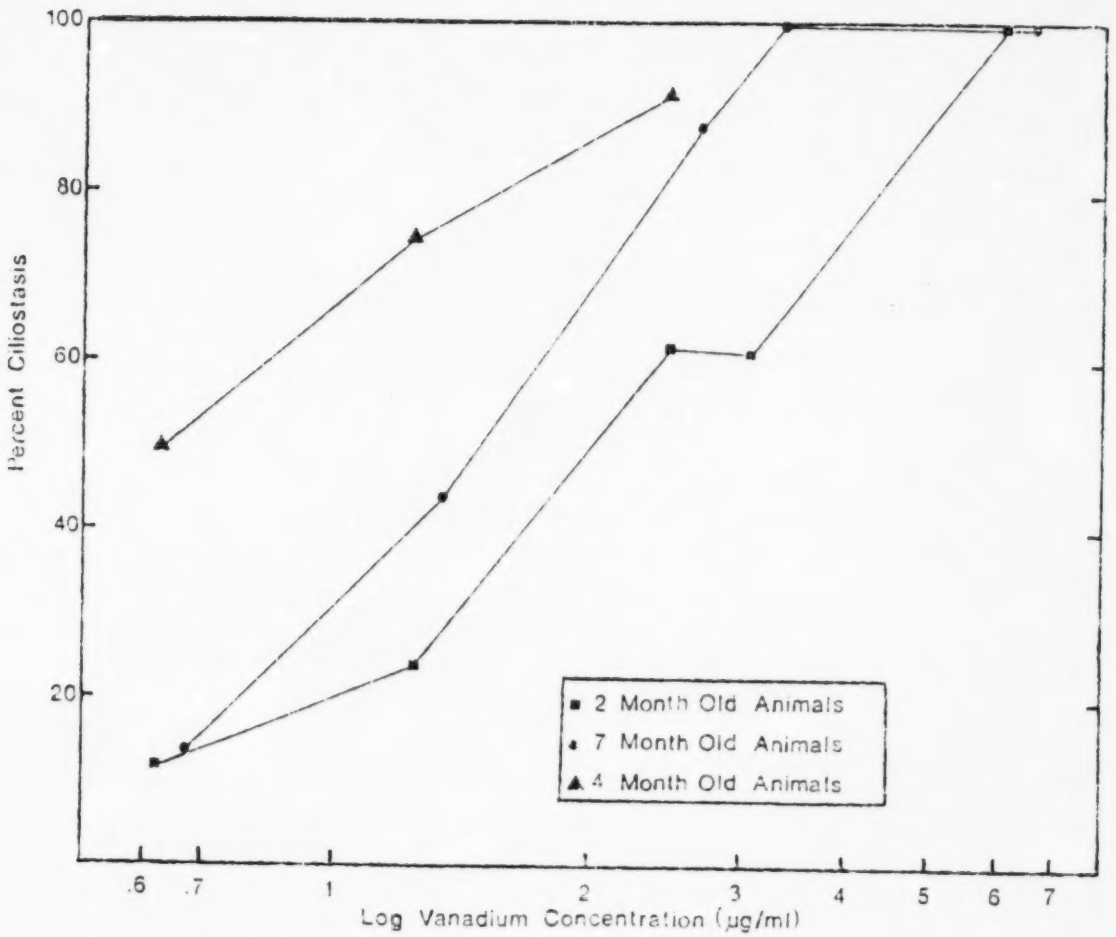

Figure 13. Percent Ciliostasis versus long concentration of Vanadium at 24 hours exposure, three studies.

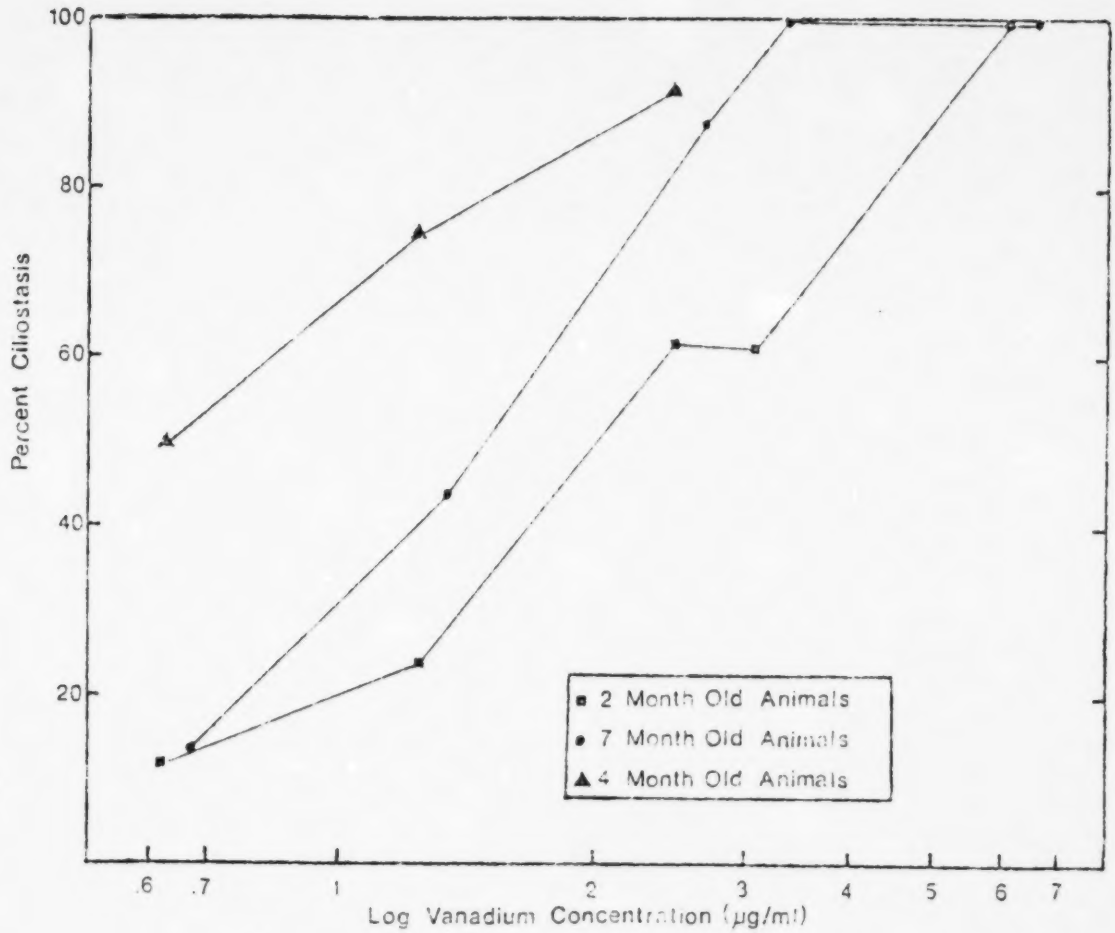

Figure 14. Percent Ciliostasis versus log concentration of Vanadium at 48 hours exposure, three studies.

To test whether the ciliostasis indicated cell death, four totally ciliostatic rings treated at 2.70 µg V/ml for 24 hours were transferred to fresh control medium. After 24 hours, beating was restored. This is validated by previous *in vivo* recovery studies (1,2).

ATP Measurements

The data for treatment with vanadium are listed in Table 6. All animals were 2-months old. The analysis shows no significant differences due to the sex of the animals and no significant differences between means due to the treatment levels. There is no interaction between the sex of the animal and treatment levels. Though the values seem to show an increase at 0.63 and 1.25 ug/ml followed by a decrease to a 12.52 µg/ml value of less than the controls, the data are not consistent enough for this to be statistically significant.

Table 6

ATP content of rings exposed *in vitro* to ammonium vanadate

Treatment (µg/ml)	Males		Females		Combined Sexes	
	ug ATP/mg fresh tissue Mean (2S.E.)					
Control	0.321	(0.072)	0.260	(0.034)	0.293	(0.202)
0.63	0.338	(0.018)	0.290	(0.056)	0.309	(0.034)
1.25	0.331	(0.058)	0.307	(0.032)	0.321	(0.050)
2.50	0.299	(0.040)	0.265	(0.032)	0.281	(0.030)
6.25	0.299	(0.052)	0.281	(0.032)	0.290	(0.034)
12.52	0.278	(0.016)	0.219	(0.034)	0.244	(0.034)

2 rings per concentration from each of 8 animals (4 males, 4 females)

ZINC

The objective of this study was to compare the toxicity of the zinc ion, the ammonium ion, and the zinc ammonium ions. The zinc ammonium sulfate was prepared stroichiometrically so that the number of zinc ions equaled the number of ammonium ions. Although there was additional sulfate in $Zn(NH_4)_2(SO_4)_2$ it was felt to be of no significance since the control medium itself contained sulfate ions (200 µg $MgSO_4$ lml).

Experiments using the ammonium ion alone were not successful because of the volatility of ammonium. The exposure procedure utilized in this study allowed the free ammonia to escape into the atmosphere of the incubator.

Analysis of Ciliostasis Effects

The percent of ciliostasis data are presented in Table 7. Within a given treatment, $ZnSO_4$ or $Zn(NH_4)_2(SO_4)_2$ almost all differences were significant. This indicated a definite dose response and a definite time response.

TABLE 7 Percent Ciliostasis of Tracheal Rings Exposed In Vitro to Various Concentrations of Zinc Sulfate or Zinc Ammonium Sulfate.

Time After Treatment	0 hours		24 hours		48 hours		72 hours
Concentration $ZnSO_4$							
Control	0		0		0		0
50 µg/ml	0		1.6		1.6	*a	*12.5
75	0	*	*40.6	*	*73.4		87.5
100	0	*	50.0	*	89.1		100.0
Concentration $Zn(NH_4)_2SO_4)_2$							
Control	0		0		0		0
50/28 µg/ml	0	*	*12.5	*	*23.4	*	*32.8
75/41	0	*	*45.3	*	*76.6	*	*90.6
100/55	0	*	*79.7	*	*95.3		100.0

[a] astericks denote significant differences ($P \leq 0.025$)

When the data are graphed as percent ciliostasis versus time (Figures 15 and 16) a significant response difference due to the higher dose levels is seen and there appears to be an increase in toxicity between 50 and 75 µg Zn with or without the ammonium ion. (Note the sharp increase in slope of ciliostasis for concentrations above 50 µg/ml.

There is also an apparent increase toxicity of $Zn(NH_4)_2(SO_4)_2$ compared to $ZnSO_4$ alone. This is more obvious at lower concentrations and the 24 hour time period. All exposures at 50 µg Zn plus 28 µg NH_4 per milliliter are significantly more toxic ($P < 05$) than exposure to Zn alone. At all time periods, 75 µg Zn plus 41 µg NH_4 per milliliter was more toxic than zinc alone, but the difference was not statistically significant. Finally, the 100 µg Zn plus 55 µg NH_4 per milliliter was significantly more toxic only at 24 hours.

ATP Analysis

The results of the ATP analysis are presented in Table 8 . They are graphed in Figure 17.

The zinc sulfate treated rings show a steady, dose-related straight line decrease in ATP content as do the zinc ammonium sulfate treated rings. However, the zinc ammonium sulfate is not quite as smooth a relationship. The ANOVA of the data showed that there were significant differences due to treatment material ($P < 0.001$), and the interaction of treatment material and level ($P < 0.001$). The zinc sulfate differed from the zinc ammonium sulfate for the 50 µg Zn - 50 µg Zn/28 µg NH_4 contrast and the 100 µg Zn - 100 µg Zn/28 µg NH_4 contrast.

Contrasting the treatment levels within a material, showed that all treatment levels caused a significantly different ATP content from their respective controls ($P = 0.001$). For both zinc sulfate and zinc ammonium sulfate, the 50 µg Zn/ml level differed significantly from the 100 µg Zn/ml level. The 75 µg Zn/ml level was not significantly different from the 50 µg Zn/ml level for either material, but did differ from the 100 µg Zn/ml at the $P = 0.05$ level for zinc ammonium sulfate.

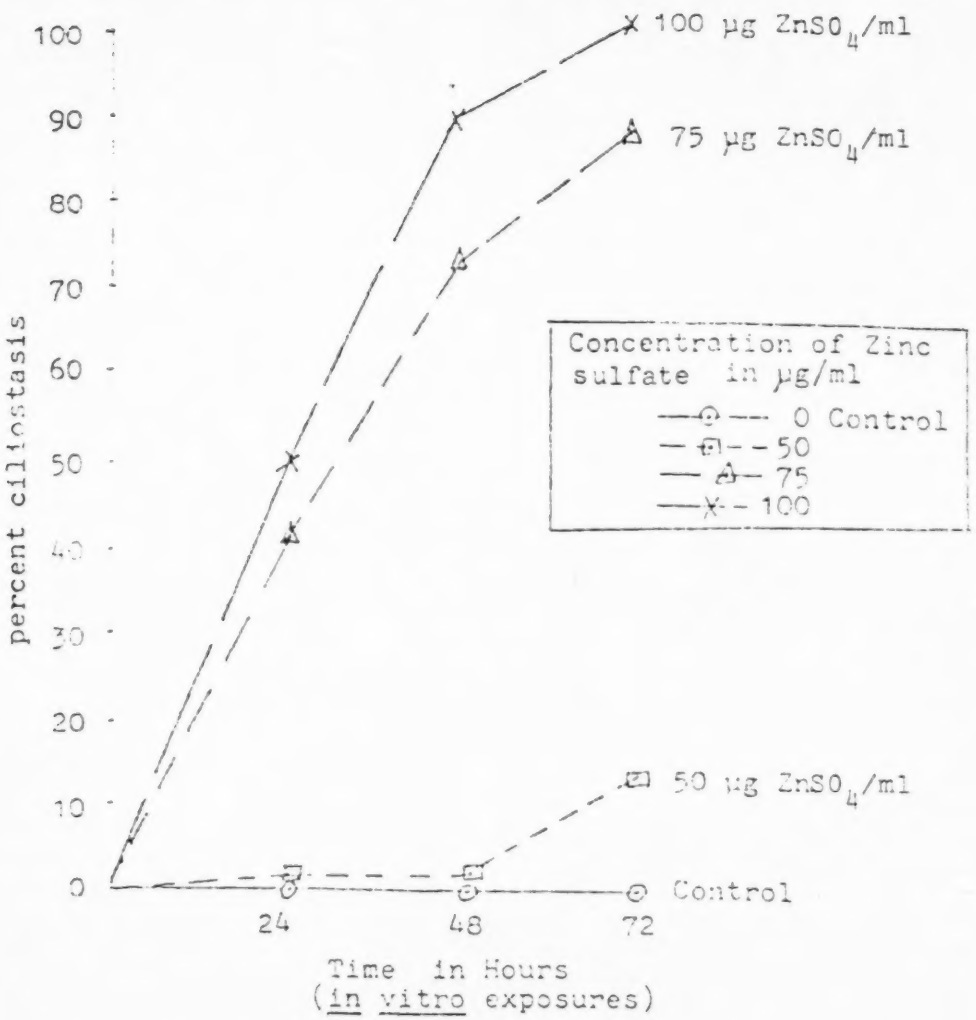

Figure 15. Percent ciliostasis versus time for three concentrations of zinc sulfate.

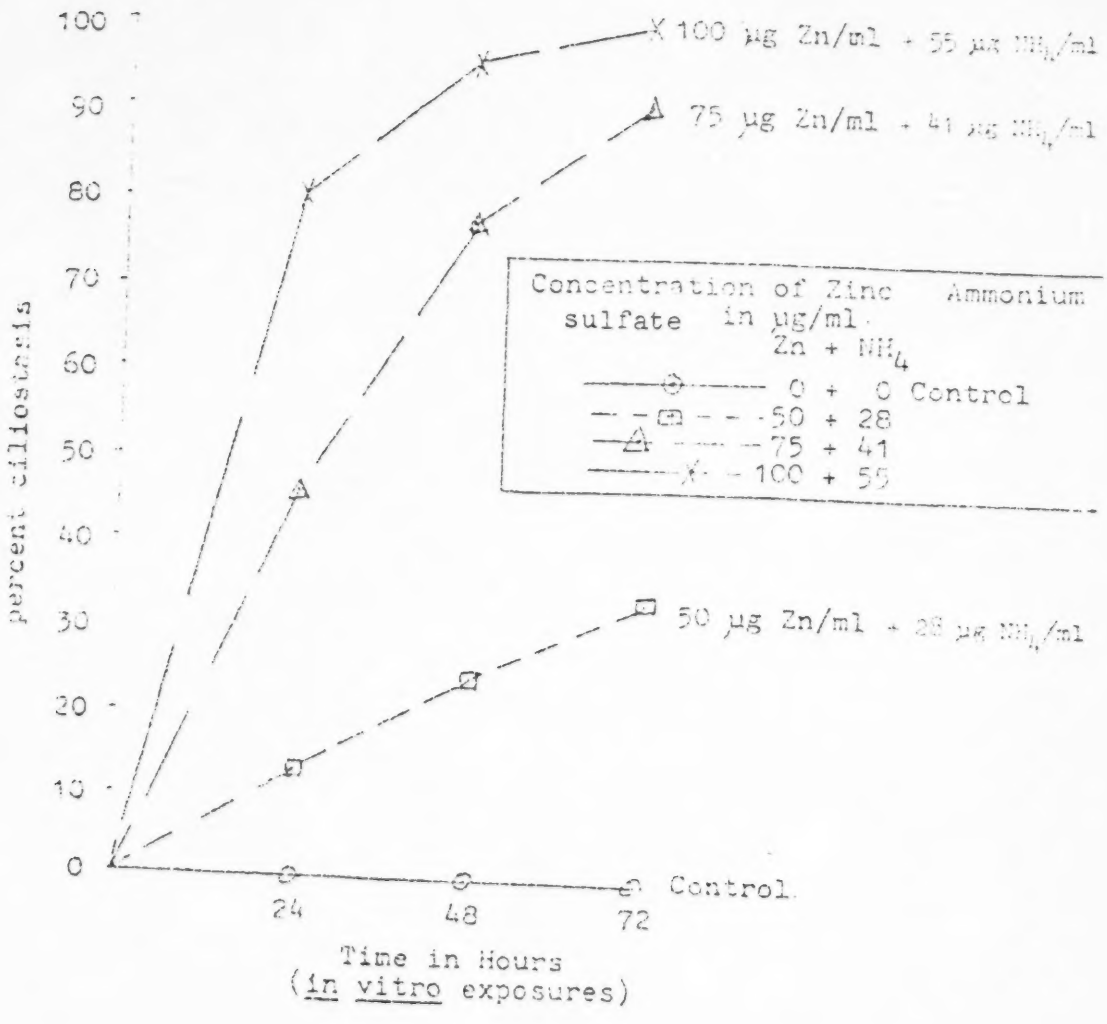

Figure 16. Percent ciliostasis versus time for three concentrations of zinc ammonium sulfate.

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Table 8. ATP CONTENT OF RINGS TREATED IN VITRO FOR 24 HOURS WITH ZINC SULFATE AND ZINC AMMONIUM SULFATE

Concentration of Zn/NH_4 (µg/ml)	ATP CONTENT OF RINGS (µg ATP/mg fresh tissue)	
	Zn^{+2} (N=24)	$Zn^{+2} + NH_4$+(N=24)
0/ 0	0.377[1]	0.351[1]
50/28	0.262 [2]	0.176
75/41	0.209	0.147 [3]
100/55	0.147 [2]	0.067 [3]

[1]All treatment levels are significantly different from their respective controls (P=0.001).

[2]ATP values connected by horizontal lines are significantly different at P=0.001

[3]Treatment levels connected by vertical line significantly different at P=0.05

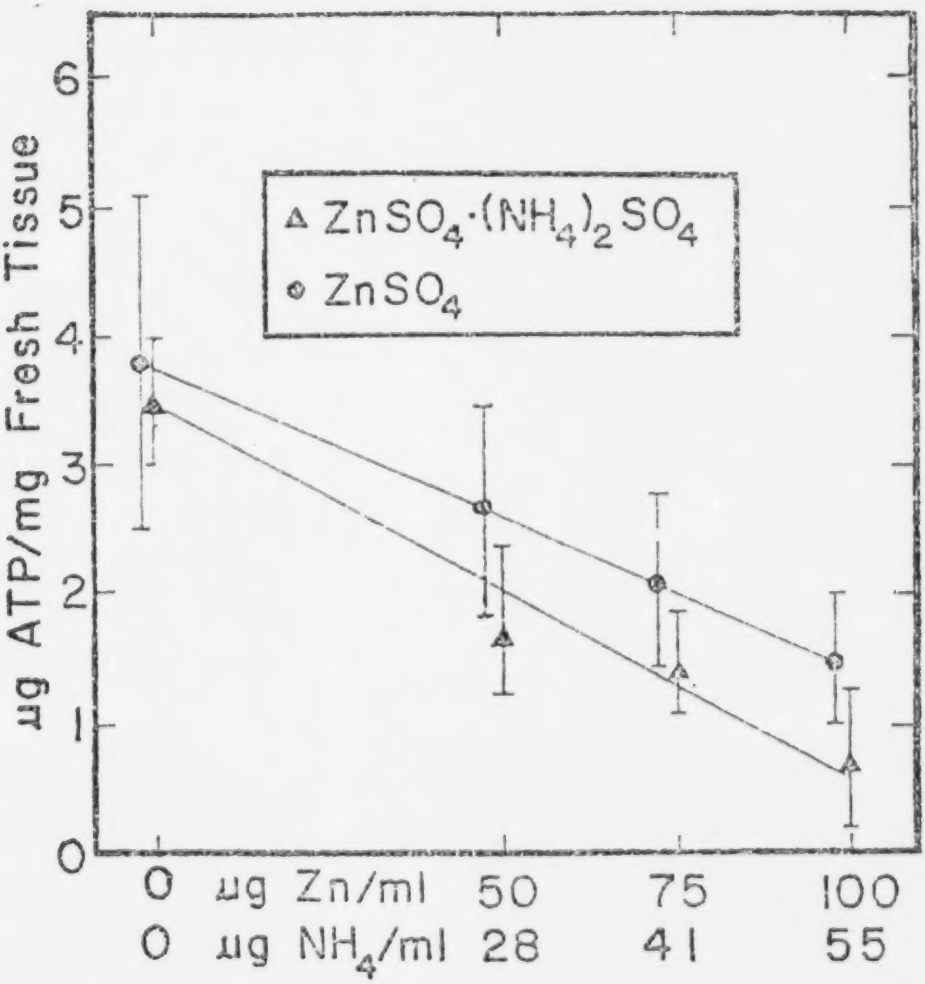


Figure 17. ATP content of tissues versus zinc sulfate or zinc ammonium sulfate concentration.

PART II. *IN VIVO* EXPOSURE TO OZONE

SECTION I

INTRODUCTION

An increase in ozone concentrations of ambient air has become one of the prime concerns of air quality organizations. Ozone, a gas, which has its chemical origin as a product of photochemical-oxidant formation, is considered a secondary pollutant since it is formed as a result of chemical reactions in the atmosphere rather than being directly emitted by a pollution source. Primary pollutants (those directly emitted by a pollution source) most responsible for ozone and other oxidant formation in the air are the nitrogen oxides, hydrocarbons, and carbon monoxide. Ozone and other oxidants formation is related to time of day, meteorologic conditions, and the amount of primary pollutants in ambient air. Ozone is used by the U.S. Environmental Protection Agency as an indicator of photochemical smog.

While virtually all studies of ozone on humans agree that ozone is harmful, the exact effect of ozone on humans is still uncertain. In controlled studies, Bates and Hazucha (1) reported a decrease in maximal flow rate at 50% of vital capacity, a decrease on maximal transpulmonary pressure, and an increase in total pulmonary resistance of humans after exposure to ozone. In these controlled studies, most subjects complained of cough, chest tightness and substantial soreness.

In another series of controlled studies with humans (2-4), high concentrations of ozone were associated with, but not the direct cause of increased mortality rates, increased hospital admissions, acute respiratory infection and aggravation of pre-existing diseases. Athletic performance has also been reported to decrease during high level concentrations of photochemical smog (5).

More recently a link between high altitude flying and ozone poisoning was suggested in a survey of airline personnel. This link was confirmed by a House subcommittee (6) on oversight and investigations, chaired by Bob Eckhardt (D-Texas, 18 July 1979).

There have been several studies on the effect of ozone on enzymes in laboratory animals. Holzman et al. (7) found that exposing mice and rabbits to ozone reduced the amount of active lysozyme, an antibacterial enzyme, normally present in large quantities in the mucous of the respiratory tract.

Palmer et al. (8) found that a single three hour *in vivo*

exposure to ozone inhibited benzypyrene hydroxylase activity in the tracheobronchial mucosa of New Zealand white rabbits. Benzypyrene hydrozylase is normally present in fairly high amounts in rabbit tracheobronchial mucosa, the site of origin, in man at least, of most primary carcinomas of the lung. There is increasing evidence that the enzyme benzypyrene hydroxylase may be of great clinical significance in detoxifying polyaromatic hydrocarbon carcinogens *in vivo*.

The pulmonary alveolar macrophage, an actively phagocytic cell, is considered to contribute to the lung's defence mechanism against both biological and chemical toxicants. A number of studies have investigated the effects of ozone, alone, or interacting with biological agents on this cell type. Hurst et al. (9) exposed New Zealand rabbits for three hours to various levels of ozone from 0.25 to 7 ppm and measured acid phosphatase, lysosome, and B-Glucuronidase and found that ozone at those levels depressed the activity of all three enzymes in isolated alveolar macrophages. Following a 24-hour *in vivo* recovery period the enzymatic activity returned almost to normal even after exposure to the highest concentration of ozone.

In a study by Coffin et al. (10) *in vivo* exposure to 5 ppm ozone produced in rabbits an influx of heterophilic leukocytes and diminution of pulmonary alveolar macrophages obtained by pulmonary lavage. These results were dose related and evident immediately after one three-hour exposures. Peak results occurred about six hours later, and were still present, but at a reduced rate, at 24 hours. They also report a marked depression in phagocytic activity following exposure to ozone. Not only were fewer bacteria ingested by the alveolar macrophages, but there was a decrease in the number of cells that were phagocytic. Some effect was noted at concentrations as low as 0.3 ppm ozone.

Gardner et al. (11) utilized a unilateral lung exposure technique to study the effects of prior ozone exposure on edema formation. Statistical analysis of the data showed that the initial ozone exposure induced a tolerance against pulmonary edema but that no protection was afforded against the toxic effects of ozone. The numbers, viability, and markedly reduced enzymatic activities of alveolar macrophages were equal in both exposed and control lungs, and inflamation, measured by the presence of polymorphonuclear leukocytes, was increased two-fold in the ozone exposed lung.

Coffin and Gardner (12) reported a synergistic mechanism of action of combined chemical and biological agents using an ozone-streptococcus model. In mice, ozone exposure preceding or following exposure to pathogenic bacteria resulted in increased mortality. The action of ozone appeared to be mediated through alteration of specific antibacterial mechanisms of the lungs, the macrophage system. In studies

with rabbits, exposure to ozone resulted in a decrease in total numbers, phagocytic, and lysosomal activities of the alveolar macrophages. The exposure to ozone inhibited bacterial clearance and also permitted uncontrolled growth of the pathogenic organisms.

Gardner et al. (13) found that the exposure to New Zealand white rabbits to ozone not only alters the types, numbers, and biochemical makeup of the pulmonary cells, but also has an adverse effect on the acellular component (LW-SI) of the lavage fluid. A protective factor found in LW-SI was degraded or destroyed by the *in vivo* action of ozone at 10 ppm for 2.5 hours. Similar results were also observed following *in vitro* exposure to 10 ppm ozone for 30 minutes.

Collier and others (14-18) have studied the effects of biological agents on the respiratory system using *Mycoplasma penumonia* and hamster tracheal organ culture. Among other effects noted, was an inhibition of protein and RNA synthesis (19). This same hamster tracheal organ culture model has also been used to study the toxic effects of environmental pollutants (chemical agents) including cadmium and nickel (19-21). Among other effects noted in these studies was a decrease in ciliary beating frequency occurring after *in vivo* and *in vitro* exposure to the metal ions.

The overall purpose of Part II of this study was to determine the effects of *in vivo* exposure to three concentrations of ozone on the ciliated epithelium of hamster tracheal rings. Specifically, two parameters were studied following *in vivo* exposures to ozone. A variety of times and doses were studied to include 0.25, 0.5, and 1.0 ppm for 3 hours for 1, 3, or 5 days.

1) A cytopathological assessment was made to determine if ozone alters or interferes with the structural integrity of the ciliated epithelium and

2) Quantitative measurements of the cilia beat frequency were determined using a stroboscope and statistically analyzed for significant differences between control and exposed rings.

SECTION 2

CONCLUSIONS

The purpose of Part II of this project was to evaluate the effects of *in vivo* exposures to ozone on the clearance mechanism of the upper respiratory tract. Alterations in ciliary function and histology are believed to be indicative of an impairment of the normal clearance mechanism.

The data from these experiments indicate the existence of a statistically significant dose related reduction in ciliary beating frequency in rings from exposed hamsters when compared with controls ($P<0.005$).

Histologically, the damage to the ciliated epithelium increased with time of exposure and concentration of ozone.

SECTION 3

RECOMMENDATIONS

Because of the widespread and increasing occurrence of ozone pollution, further studies should be conducted. The short term acute exposures to ozone in this study should be extended to include ozone in combination with other pollutants. Also needed are studies to determine the effects of ozone in combination with stress factors such as exercise or in relation to diet.

Other studies recommended include the following:

1. Recovery of ciliary activity following *in vivo* exposures to ozone.

2. Measurement of ATP production in hamster heart, liver and gastrocnemius muscle following *in vivo* exposure to ozone.

3. The effect of ozone on the oxidative capacity of hamster heart, liver and muscle.

4. Effect of ozone on the oxidative capacity of hamster tissues following exercise.

SECTION 4

MATERIALS AND METHODS

ANIMALS

Male and female Syrian Golden hamsters (outbred Lak: LVG (SYR) Charles River Lakeview, Wilmington, MA) weighing 90 to 160 g were used in these studies. Hamsters of this weight are considered young adults and are two months old or older. Animals of one sex were housed in groups of three per cage, and provided food and water ad libitum.

IN VIVO EXPOSURES

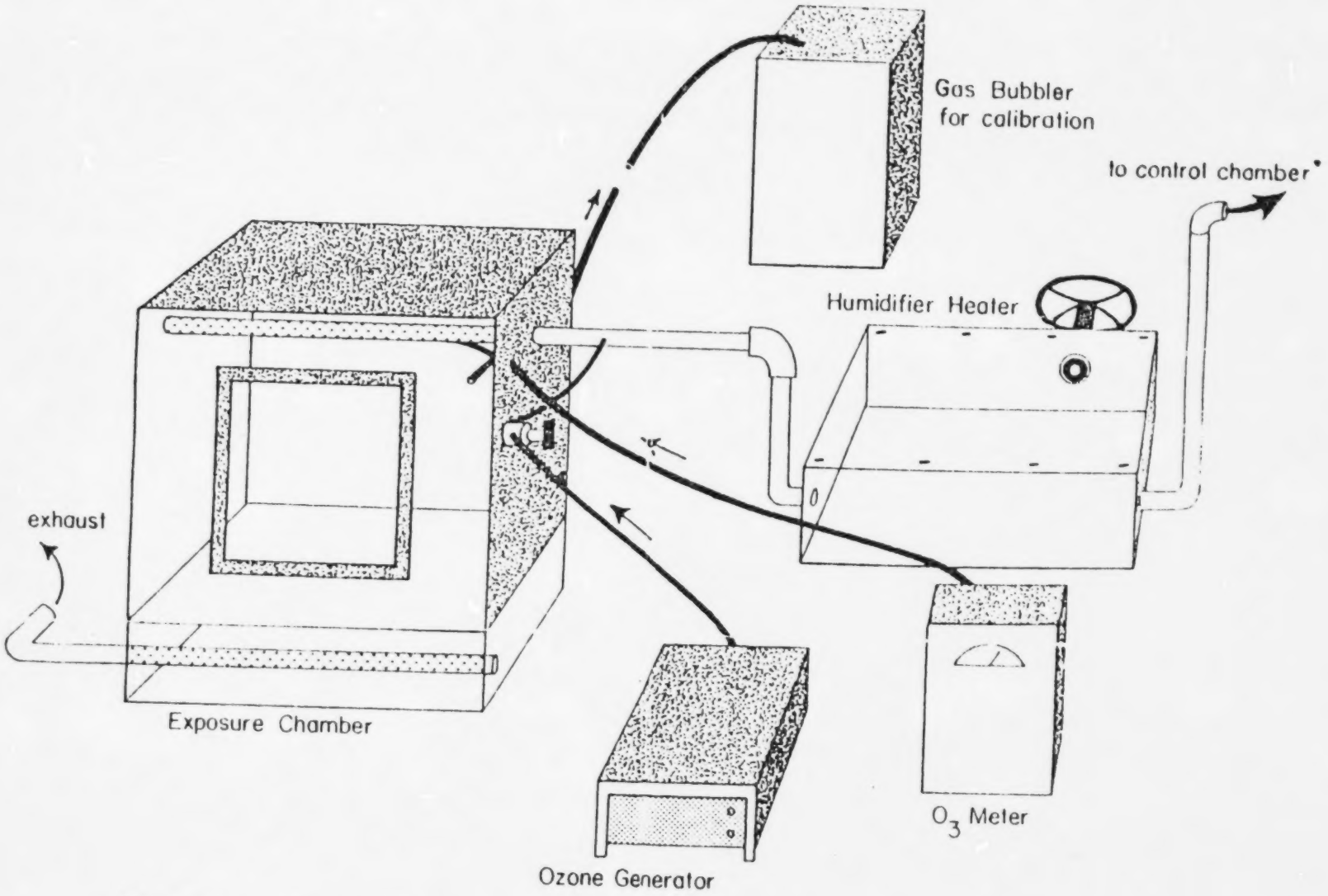
Ozone exposures were conducted in air pollution exposure chambers maintained by the Department of Natural Resources, Ball State University (Figure 1). Ozone was produced for exposure purposes by an electric arc laboratory ozone generator. Precise control of ozone levels within the chamber was attained by the use of a precision, chemically resistant, needle valve. Ozone concentrations were monitored colorimetrically with a Mast oxidant meter. The oxidant meter is calibrated daily by withdrawing ozone samples from the exposure chamber through a Potassium Iodide - Boric Acid solution (gas bubbler). Oxidant levels in these calibration samples were determined colorimetrically in a spectrophotometer.

Male and female Golden Syrian hamsters were randomly selected in equal numbers for exposure to ozone at 0.25, 0.5, or 1.0 for 3 hours times 1, 3, or 5 days. A second group of male and female hamsters in equal numbers were selected as a control group.

In all procedures, control animals were treated identically to exposed animals with exception to ozone exposure.

PREPARATION OF TRACHEAL RINGS

The method used for maintaining the tracheal rings as bio-indicators of metal toxicity has been previously described by the author (20, 21). Immediately following the final three-hour exposure period, trachea were excised aseptically, washed in Eagle's basal medium (Gibco, Grand Island, NY), and cut into rings approximately 1 mm thick. Twelve tracheal rings were usually obtained from each animal. The rings were placed in individual 50 x 9 mm wettable plastic petri dishes with 2.5 ml of Eagle's basal medium with Earle's Salts. The medium was supplemented with 10% fetal calf serum, 1% penicillin (10,000 units per ml) - streptomycin (10,000 mg per ml),



*Control Chamber identical to exposure chamber except lacks O_3

Figure 1. In vivo exposure facilities.

0.5% l-Glutamine (200 mM) and 1% mycostatin (10,000 units per ml). The pH of the medium was adjusted to 7.4 with 1N NaOH. Temperature of the medium was 37°C.

PARAMETERS MEASURED

Determination of Beat Frequency

Immediately following the tracheotomy (time equals "0-hour"), all tracheal rings were examined for normal ciliary movement and/or evidence of cytopathology with an inverted microscope at a magnification of 160 X or 250 X. Each ring was viually divided into theoretical quadrants. Ciliary beating frequency for each quadrant of a ring was determined using an inverted microscope (160 X or 250 X) with an attached electronic stroboscope (General Radio Strobotac Type 1531-AB). A Zeiss air stream incubator was utilized to maintain temperature of the ring at 37°C.

Means of beat frequency and standard deviations were computed for each treatment. In order to identify possible influence of sex differences, calculations were made from the rings of the two sexes, separately as well as combined. Complete statistical analysis of all the beat frequency data was made using an analysis of variance.

Histological Procedures

Tracheal rings were fixed in Bouin's solution immediately following excision from exposed or control hamsters. The rings were dehydrated in three changes of 50% ethanol followed by three changes of dioxane. The rings were infiltrated in three changes of Paraplast Plus *in vacuo* at 60°C, the final change always lasting overnight. After embedding in fresh Paraplast Plus, tissues were sectioned at 4 µm and stained with Harris' hematoxylin and eosin.

Photomicrography Procedures

Photographs were taken using an American Optical Microstar Series 10 microscope with an AO Expostar shutter control. All photographs were taken at a magnification of 1000X (oil).

Both black and white prints and color prints and transparencies were taken. The following films were utilized: Polaroid black and white TYPE 107, Polaroid Type 55 P/N, Polacolor Type 108 and 35-mm black and white (Kodak Plus X-Pan) and color (Kodachrome 25, Kodak Photomicrography Film #2483, and Ektachrome Tungsten 50). All black and white photographs were taken using a Wratten filter No. 50 (green). Color photographs were taken using appropriate light balancing filters (Wratten Nos. 80A, 82A and/or AO #608).

Statistical Methodology

The data were analyzed by the analysis of variance (ANOVA) program, BMDP2V, from the Health Science Computing Facility at the University of California at Los Angeles. Analyses was made for possible effects due to the different treatment materials, sex of the animal, treatment level, and their possible interactions. Where no effect was seen (between the various series of treatments, and between the sexes) the data were pooled and the ANOVA rerun. Where significant effects were seen, Scheffe's test (4,5) was used to find the significant differences. For the tests between materials at a given level a hybrid error term (6) was used. As a conservative estimate for the degrees of freedom, the lower of the two degrees of freedom of the two error terms in the hybrid was used.

SECTION 5

RESULTS AND DISCUSSION

BEAT FREQUENCY

In vivo exposure to 0.25 and 0.5 ppm ozone 3 hrs X 3 days resulted in a significant reduction in ciliary beat frequency. This reduction in beat frequency increased as the concentration of ozone increased (Figure 2). The mean beating frequency in beats per minute for 94 control rings (8 animals, 4 of each sex) was 1174.6. The mean beating frequency for 93 rings from 8 animals exposed to 0.25 ppm ozone 3 hours X 3 days was 1089.2. Mean beat frequency for 8 animals exposed to 0.5 ppm ozone was 1009.0 (89 rings). An analysis of variance indicated a significant decrease ($P<0.005$) in beat frequency in the ozone treated rings when compared with controls. There was no significant difference in beat frequency between sexes or due to interaction of sex and treatment.

The results indicate an altered functioning of the tracheal ciliated epithelium which would possibly interfere with the host's normal pulmonary clearance mechanism. This in turn would make the host more susceptible to respiratory infection.

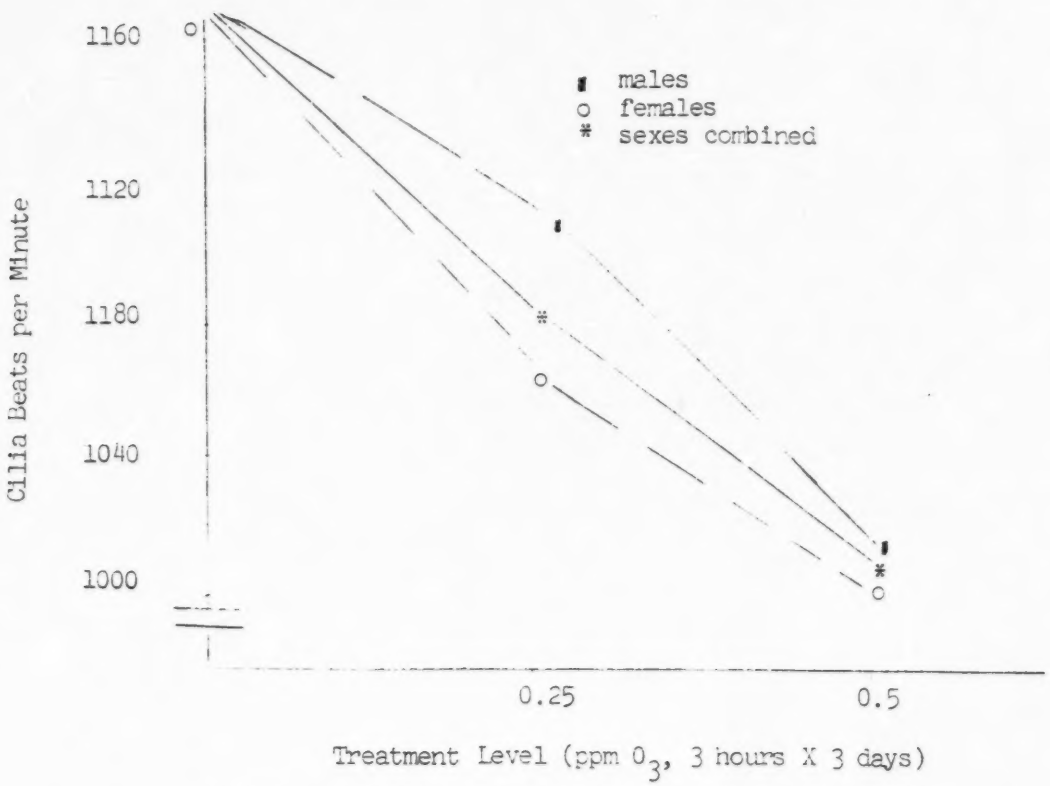


Figure 2. Ciliary beating frequency versus in vivo ozone concentration for 3 hours X 3 days. Means of four male and four female animals, sexes separate and combined. All treatment means are significantly lower than the control means ($P<0.005$).

HISTOLOGICAL OBSERVATIONS

Control Rings

The appearance of the control tracheal rings at 0-hours is shown in Figure 3. Tissue lining the lumen is a typical pseudostratified columnar, ciliated epithelium. The surface of the epithelium is relative even although a few cells are being sloughed off. The cilia appear even in length, straight, and thick with few if any signs of areas of denudation. The nuclei of the columnar cells are oval in shape and approximately centered in the cytoplasm. The cytoplasm is uniform in appearance.

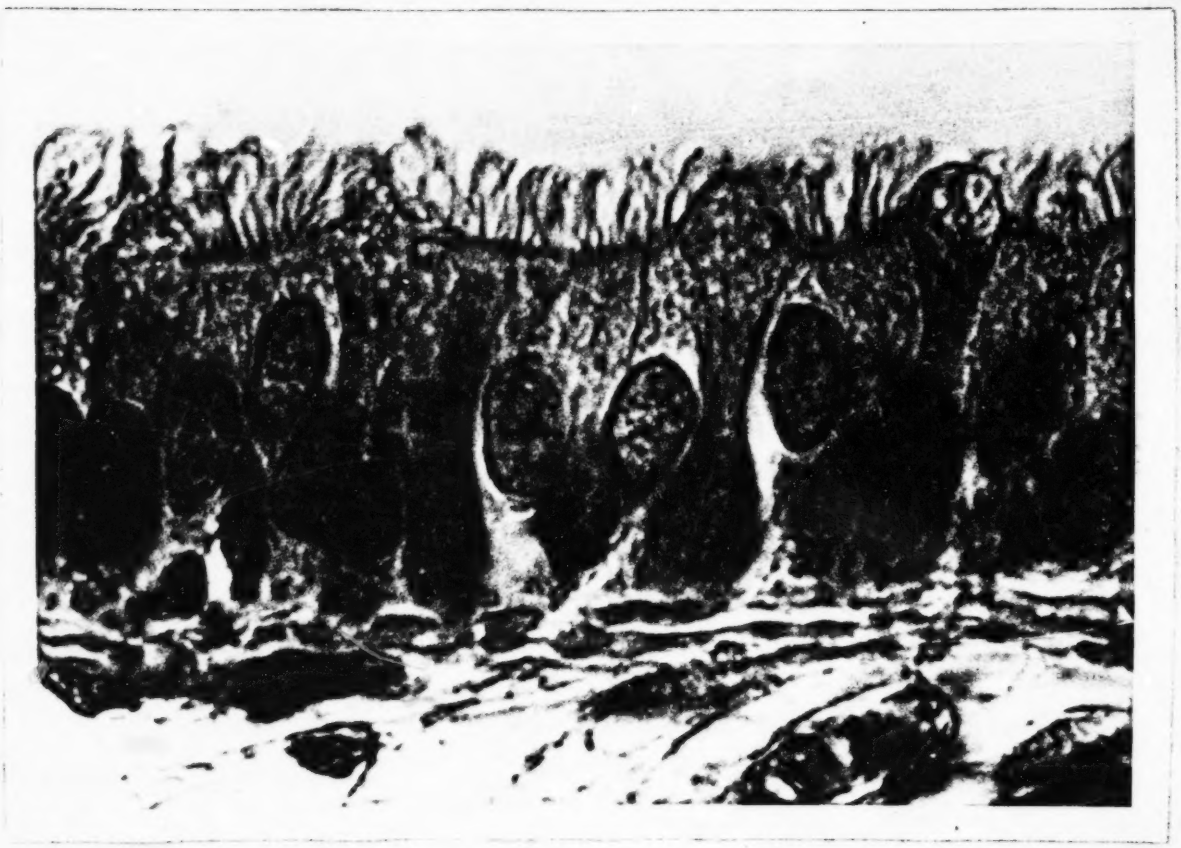


Figure 3. 0-hour old untreated ring, hematoxylin and eosin, 100X. Note the pseudostratified arrangement of columnars and basal cells. Cilia are straight, evenly distributed, and thick. Nuclei are oval in shape.

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Changes Due to Treatment with 0.25 ppm Ozone, 3 hours X 3 days.

The most obvious change following *in vivo* exposure to 0.25 ppm ozone, 3 hours X 3 days, is in the shape of the nuclei (Figure 4). Instead of oval-shaped nuclei, many are more rounded in shape and/or have flattened surfaces. The surface of the epithelium is not as smooth and even as in the 0-hour control. The cilia are more irregular, some tufting and denudation are present.

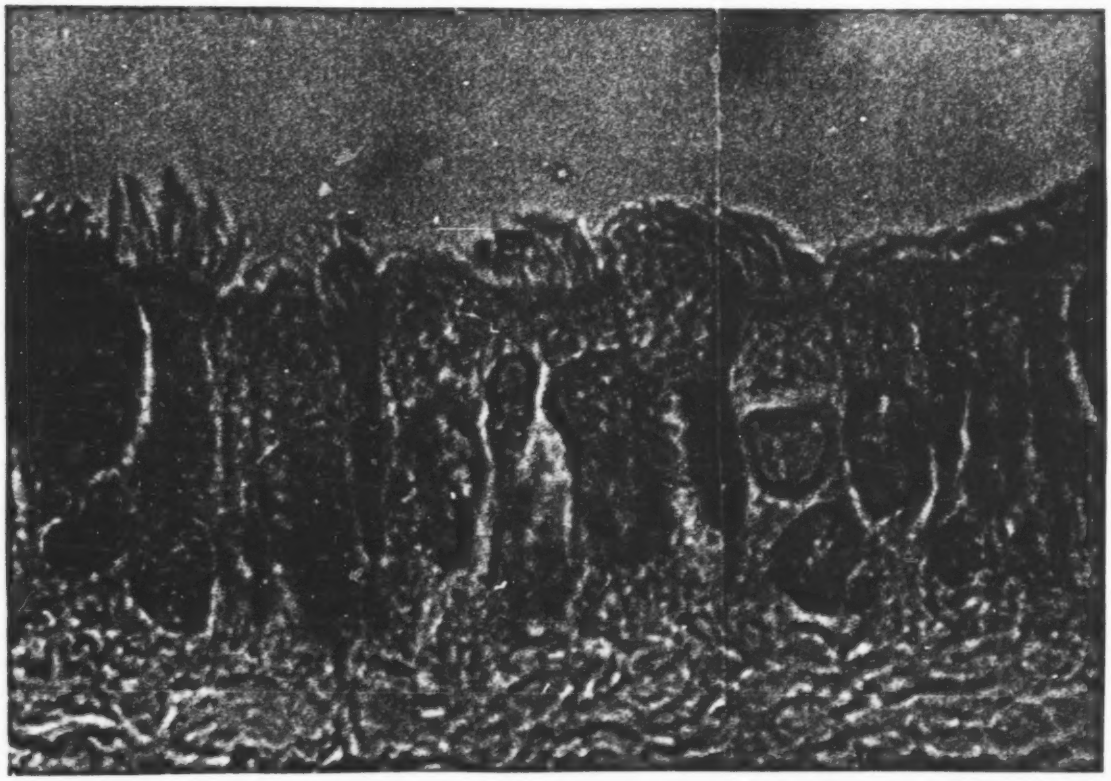

Figure 4. 0-hour ring treated *in vivo* with 0.25 ppm ozone for 3 hours X 3 days, hematoxylin and eosin, 1000X. Note rounded nuclei and irregularly arranged cilia.

Changes Due to Treatment with 0.5 ppm Ozone, 3 hours X 1 day.

After one day of *in vivo* exposure to 0.5 ppm ozone (Figure 5), the tracheal epithelium appears more like that of the controls than the rings exposed to 0.25 ppm ozone for 3 hours X 3 days. Nuclei are oval, the surface of the epithelium is relatively even, and cilia are abundant. There is, however, more indication of sluffing of cells in this exposed ring than in the control.

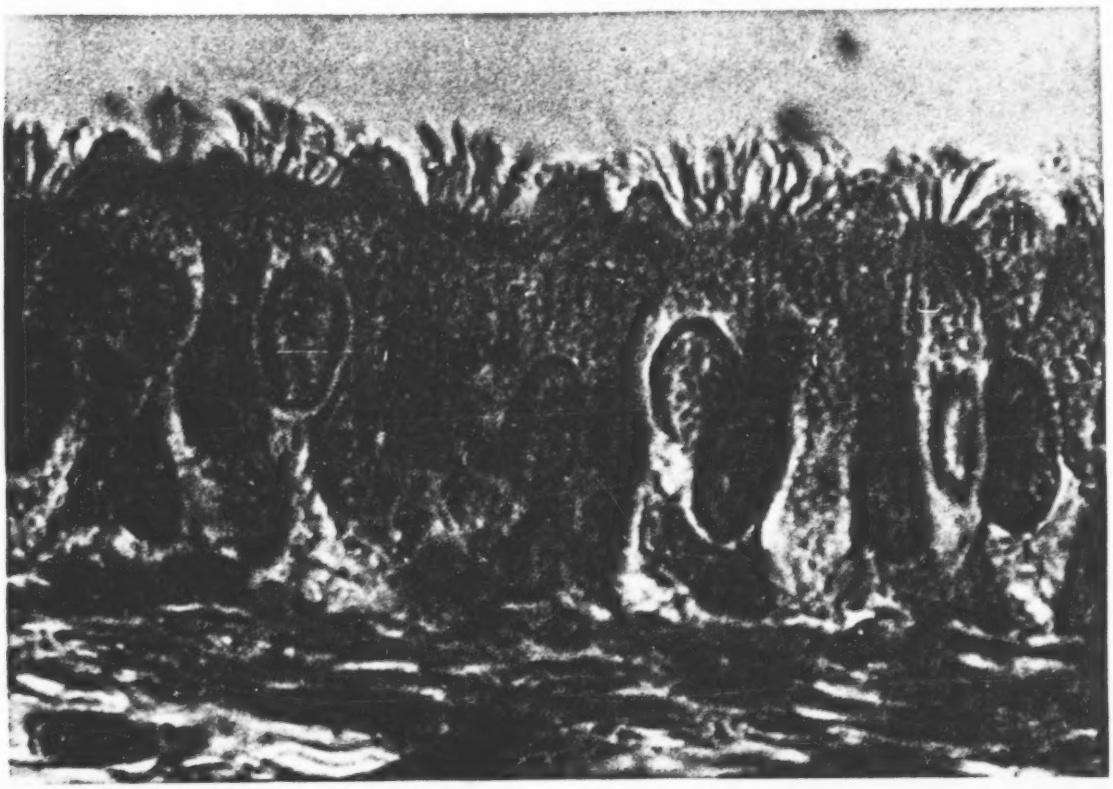

Figure 5. 0-hour ring treated *in vivo* with 0.5 ppm ozone for 3 hours X 1 day; hematoxylin and eosin, 1000X. Pseudostratified ciliated columnar epithelium similar to that of control rings. More cells being sluffed off here than in control rings. Some tufting.

Five days of in vivo exposure to 0.5 ppm ozone results in considerable damage to the tracheal epithelium. As shown in Figure 6, there is a decrease in the height of the epithelium. Cells are more cuboidal than columnar. Nuclei are rounded. Many areas of denudation are present.

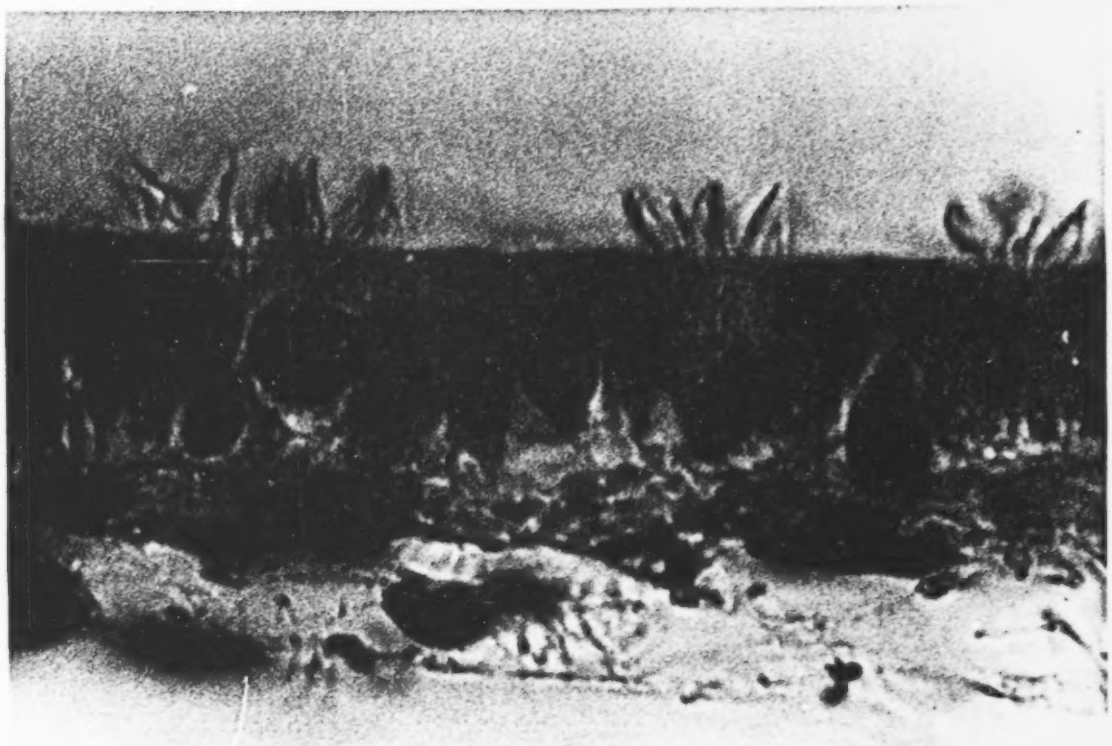

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Figure 6. 0-hour ring treated in vivo with 0.5 ppm ozone, 3 hours X 5 days, hematoxylin and eosin, 1000X. Note flattening of epithelial layer, loss of cilia, rounded nuclei.

Changes Due to Treatment with 1.0 ppm Ozone, 3 hours X 1 day.

One three-hour *in vivo* exposure to 1.0 ppm ozone produced changes in the organization of the tracheal epithelium as shown in Figure 7. The surface is very uneven with many cells sluffed off or in the process of being sluffed off. Nuclei are more rounded than in the control rings. Cilia arrangement is irregular. Tufting occurs as well as denudation.

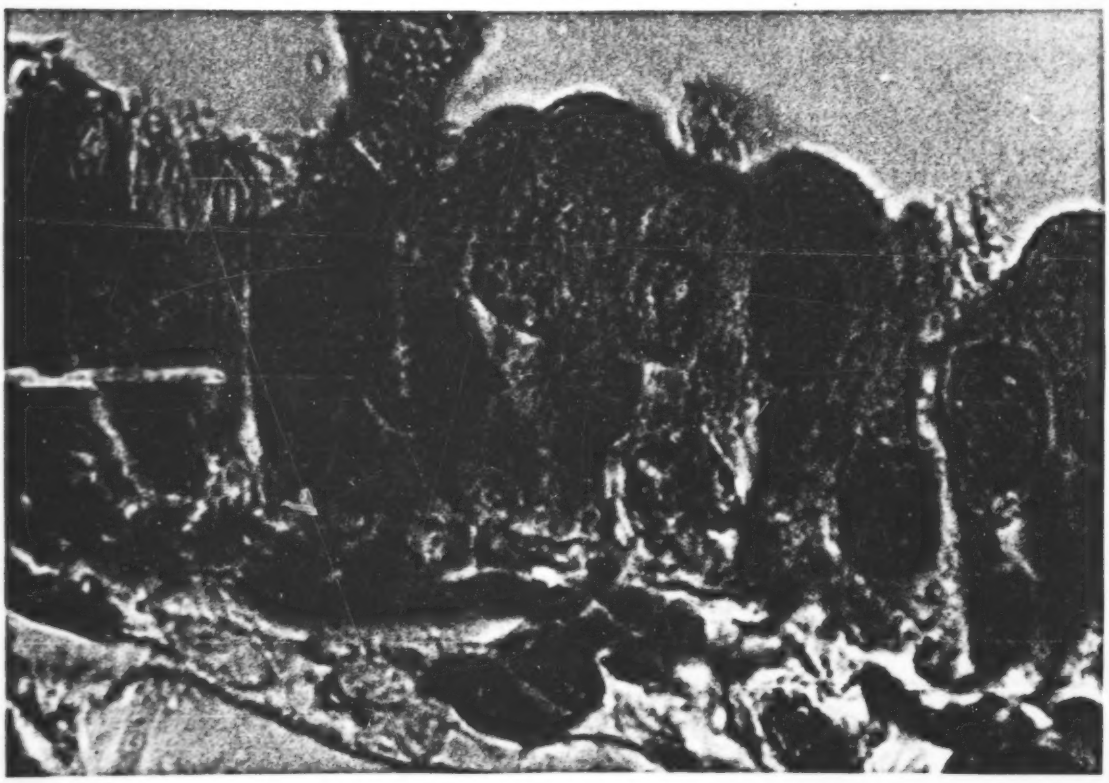

Figure 7. 0-hour ring treated *in vivo* with 1.0 ppm ozone, 3 hours X 1 day, hematoxylin and eosin, 1000X. Note irregular surface of epithelium, rounded nuclei, and loss of cilia.

Effects of *in vivo* exposure to ozone produced pathological effects which were dose related. Increasing the concentration of ozone results in increasing disorganization of the pseudostratified ciliated epithelium lining of the tracheal rings. The degree of disorganization was also directly related to the length of time or exposure at any one concentration level.

The resulting damage to tissue morphology due either to concentration or length of exposure to ozone would alter the normal functioning of the mucociliary escalator. This impairment could increase the susceptibility of the host to respiratory infections.

REFERENCES

PART I

1. Adalis, D., D. E. Gardner, F. J. Miller, and D. L. Coffin. 1977. Toxic effects of cadmium on ciliary activity using a tracheal ring model system. Environ. Res. 13:111-120.

2. Adalis, D., D. E. Gardner, and F. J. Miller. 1978. Cytotoxic effects of nickel on ciliated epithelium. Am. Rev. Respirat. Disease. 118:347-354.

3. Conover, W. J. 1971. Practical Nonparametric Statistics, Wiley, New York.

4. Dupont Biometer Manual. 1970.

5. Kirk, R. E. 1968. Experimental Design Procedures for Behavioral Sciences. McGraw-Hill,New York.

6. Scheffe, H. 1959. The Analysis of Variance. Wiley, New York.

7. Siegel, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

8. Winer, B. J. 1971. Statistical Principals in Experimental Design, 2nd ed. McGraw-Hill, New York.

PART II

1. Bates, D. and M. Hazucha. 1973. The short term effects of ozone on the human lung. pp. 507-540 In: National Research Council. Assembly of Life Sciences. Proceedings of the Conference on Health Effects of Air Pollutants, October 3-5, 1973. U.S. Senate Committee on Public Works, Print Serial No. 93-15. Washington, D.C. U.S. Government Printing Office. 1973.

2. California Department of Public Health. 1955. Clean Air for California. Initial Report of the Air Pollution Study Project. Berkeley: California Department of Public Health. March 1955. 57 pp.

3. California Department of Public Health. 1956. Clean Air for California. Second Report of the California Department of Public Health. Berkeley: California Department of Public Health, March 1956. 23 pp.

4. California Department of Public Health. 1952. Report III...A Progress Report of the California Department of Public Health. Berkeley: California Department of Public Health. Feb. 1957. 32 pp.

5. Wayne, W. S., P. F. Wehrle, and R. E. Carroll. 1967. Oxidant air pollution and athletic performance. J.A.M.A. 199:901-904.

6. Broad, W. J. 1979. High anxiety over flights through ozone. Science 205:767-769.

7. Holzman, R. S., D. E. Gardner, and D. L. Coffin. 1968. In vivo inactivation of lysozyme by ozone. Journal of Bacteriol 96:1562-1566.

8. Palmer, M. S., R. W. Exley, and D. L. Coffin. 1972. Influence of pollutant gases on benzpyrene hydroxylase activity. Arch. Environ. Health 25:439-442.

9. Hurst, D. J., D. E. Gardner, and D. L. Coffin. 1970. "Effect of ozone on acid hydrolases of the pulmonary alveolar macrophage". Journal of Reticuloendothelial Society 8:288-300.

10. Coffin, D. L., D. E. Gardner, R. S. Holzman, and F. J. Wolock. 1968. Influence of ozone on pulmonary cells. Arch. Environ. Health 16:633-636.

11. Gardner, D. E., T. R. Lewis, S. M. Alpert, D. J. Hurst, and D. L. Coffin. 1972. The role of tolerance in pulmonary defense mechanisms. Arch. Environ. Health 25:432-438.

12. Coffin, D. L., and D. E. Gardner. 1972. Interaction of biological agents and chemical air pollutants. Occup. Hyg. 15:219-234.

13. Gardner, D. E., E. A. Pfitzer, R. T. Christian, and D. L. Coffin. 1971. Loss of protective factor for alveolar macrophages when exposed to ozone. Arch. Intern. Med. 127:1078-1084.

14. Collier, A. M., W. A. Clyde, and F. W. Denny. 1969. Biological effects of Mycoplasma pneumoniae and other mycoplasmas from man on hamster tracheal organ culture. Proc. Soc. Exptl. Biol. Med. 132:1153-1158.

15. Collier, A. M., W. A. Clyde, and F. W. Denny. 1971. Mycoplasma pneumoniae in hamster tracheal organ culture: immunofluorescent and electron microscopic studies. Proc. Soc. Expt. Biol. Med. 136:569-573.

16. Collier, A. M., and J. B. Baseman. 1973. Organ culture techniques with mycoplasmas. Ann. N.Y. Acad. Sci. 225:229-289.

17. Hu, P. C., A. M. Collier, and J. B. Baseman. 1975. Alterations in the metabolism of hamster tracheas in organ culture after infection by virulent Mycoplasma pneumoniae. Infection and Immunity 11:704-710.

18. Gabridge, M. G., and R. B. Polisky. 1972. Intracellular levels of adenosine triphosphate in hamster tracheal organ cultures exposed to Mycoplasma pneumoniae cells or membranes. In Vitro 13:510-516.

19. Cross, C. E., A. B. Ibrahim, M. Ahmed, and M. G. Mustafa. 1970. Effect of cadmium ion on respiration and ATPase activity of the pulmonary alveolar macrophage. Environ. Res. 3:512-520.